610.5
A595
M497
v.31
no.2

ANNALES MEDICINAE EXPERIMENTALIS ET BIOLOGIAE FENNIAE

REDACTORES:

E. MUSTAKALLIO (TURKU)
U. UOTILA (HELSINKI)
ARMAS VARTIAINEN (HELSINKI)
ALVAR WILSKA (HELSINKI)
A. I. VIRTANEN (HELSINKI)

EDITOR

K. O. RENKONEN

REDIGENDOS CURAVIT

ODD WAGER

VOL. 31 1953 FASC. 2

MERCATORIN KIRJAPAINO
HELSINKI, FINLAND

UNIVERSITY OF MICHIGAN
MEDICAL LIBRARY

Annales Medicinae Experimentalis et Biologiae Fenniae

is a direct continuation of the *Acta Societatis Medicorum Fennicae* »*Duodecim*», 1919—1930 (Vols. I—XII) and the *Acta Societatis Medicorum Fennicae* »*Duodecim*», *Ser. A*, 1931—1946 (Vols. XIII—XXIV).

The journal is published by the Finnish Medical Society »Duodecim» with the object of providing an opportunity to publish articles on experimental medicine and on related biological subjects.

Each number of the journal contains 80—100 pages, three numbers forming one volume. Articles are accepted for publication with the understanding that they are original contributions never previously published. The manuscripts should be in English, French or German, and typewritten. They should not exceed a total length of 16 pages, and there should be a short summary at the end of the article.

The subscription price is Fmks 750 per volume in Finland and $ 4 or Swedish Crowns 15.00 in foreign countries. More extensive works, published as supplements, the subscribers receive free of charge.

Address for subscription, exchange of reviews, and all communications:

Annales Medicinae Experimentalis et Biologiae Fenniae

Yrjönkatu 17, Helsinki, Finland.

Annales Medicinae Experimentalis et Biologiae Fenniae

est une suite directe des revues *Acta Societatis Medicorum Fennicae* »*Duodecim*», 1919—1930 (V. I—XII) et *Acta Societatis Medicorum Fennicae* »*Duodecim*», Ser. A, 1931—1946 (V. XIII—XXIV).

La revue est éditée par la Société de médecins finnois »Duodecim», et a pour but d'offrir l'occasion de publier des recherches scientifiques appartenant à la médicine expérimentale et à la biologie étroitement liée avec la médicine.

La revue paraît en cahiers comprenant à peu près 80—100 pages. 3 cahiers forment un volume. Les articles destinés à la revue ne doivent pas être publiés ailleurs. Les manuscrits dactylographiés doivent être rédigés en français, allemand ou anglais et leur longeur totale ne doit pas en général dépasser 24 pages. Un court résumé doit se trouver à la fin.

Le prix de l'abonnement pour la Finlande est marcs 750, pour l'étranger $ 4 ou couronnes suédoises 15 par volume. Les travaux plus étendus, qui seront éventuellement publiés en suppléments, seront distribués gratuitement aux abonnés.

Adresse pour abonnement, échange de journaux et toutes autres communications:

Annales Medicinae Experimentalis et Biologiae Fenniae

Yrjönkatu 17, Helsinki, Finlande.

Annales Medicinae Experimentalis et Biologiae Fenniae

sind eine direkte Fortsetzung der *Acta Societatis Medicorum Fennicae* »*Duodecim*», 1919—1930 (Vol. I—XII) und der *Acta Societatis Medicorum Fennicae* »*Duodecim*», *Ser. A*, 1931—1946 (Vol. XIII—XXIV).

Die Zeitschrift wird vom *Finnischem Ärzteverein* »*Duodecim*» herausgegeben und hat zur Aufgabe wissenschaftliche Untersuchungen aus dem Gebiete der experimentellen Medizin und sich daran schliessenden biologischen Forschungsgebieten aufzunehmen.

Jede Nummer der Zeitschrift erscheint in einem Umfange von 80—100 Druckseiten. Drei Nummern bilden ein Volumen. In die Zeitschrift werden nur Originalarbeiten aufgenommen, die nicht früher veröffentlicht worden sind. Die mit Schreibmaschiene geschriebenen Manuskripte sind in deutscher, englischer oder französischer Sprache einzusenden und sollen im allgemeinen nicht mehr als 24 Druckseiten betragen. Jede Untersuchung ist durch eine kurze Zusammenfassung abzuschliessen.

Der Bezugspreis beträgt für das Innland 750 Fmk und fürs Ausland 4 $ bzw 15 Schwedenkr. Umfangreichere Untersuchungen, die als Supplemente herausgegeben werden können, erhalten die Abonnenten abgabefrei.

Bezugsadresse sowie Anschrift für Austausch von Zeitschriften und alle anderen Mitteilungen:

Annales Medicinae Experimentalis et Biologiae Fenniae

Yrjönkatu 17, Helsinki, Finnland.

610.5
A595
M497
v. 31
no. 2

FROM THE INSTITUTE OF OCCUPATIONAL HEALTH (M.J.K. & M.E.P.), HELSINKI,
AND FROM THE CHILDREN'S CLINIC, UNIVERSITY OF HELSINKI (V. L.)

THE EFFECT OF PITUITRIN, PITRESSIN, AND PITOCIN ON URINARY SODIUM AND POTASSIUM EXCRETION IN DOG

by

M. J. KARVONEN, V. LEPPÄNEN, and M. E. PITKÄNEN

(Received for publication October 25, 1952)

Earlier investigators have published discrepant findings concerning the effect of posterior pituitary hormone and of its fractions on the electrolyte excretion in urine of different experimental animals (1, 2, 3, 4, 5, 6, 8, 9, 10, 12, 14, 15, 16, 17, 18, 19, 20). This gave the present writers a stimulus to subject the problem to a new study with somewhat different methods. In the present work, the effect on the excretion of water, sodium, and potassium of the postpituitary extract and its fractions was observed. For the elimination of the body's own pituitrin production, the experiments were performed during maximum water diuresis. The use of a flame photometer rendered possible the analysis of small samples of urine, collected at short intervals.

METHODS

Two trained mongrel bitches were used for the experiments. Before each experiment the dog was fasted overnight. Prior to the beginning of the experiment, the dog was prehydrated with 30 cc per kg of body weight of water or 0.45 per cent NaCl, in order to bring the animal to a standard state. Half of the experiments were made using water, and the other half with NaCl solution. After 1½

hours the dog was given the same amount of water, it was catheterized, and the urine samples were collected at the intervals of two minutes. When the urine flow had reached a high and reasonably stable level, 0.25 or 1.0 milliunits (mU) of pituitrin, pitressin, or pitocin (Parke & Davis) was injected intravenously and the collection of urine samples was continued, until the antidiuresis was passed. With 0.25 mU of pituitrin or pitressin — which is a threshold dose for eliciting an antidiuretic response — antidiuresis was not observed in all experiments. In the samples, sodium and potassium content was determined with a flame photometer. During the course of the work, two flame photometers of own design were used (11 & 13). Control experiments without any injections were also performed.

RESULTS

Four *control* experiments showed that no marked spontaneous changes occurred in the excretion of sodium and potassium during water diuresis in these dogs.

Pituitrin. — In five of seven experiments, 0.25 mU of pituitrin produced a clearcut antidiuresis. In four experiments with 1.0 mU a pronounced antidiuresis regularly ensued. During the antidiuresis there always occurred either an absolute rise of the *sodium* excretion over the pre-injection level or a relative rise, following a primary fall, without, however, exceeding the pre-injection level. A typical example of the former type of results is shown in Fig. 1. In most experiments, the *potassium* excretion behaved in an analogous way, but a rise in the potassium excretion did not occur with the same regularity as with sodium. The rise in the sodium and potassium excretion coincided in most experiments with the passing of the maximum antidiuresis, but in a few experiments a rise of the electrolyte excretion started already with the onset of the antidiuretic response. The results of each experiment are summarized in Table I.

The *Na/K relation* in the urine did not show any consistent effects after 0.25 mU of pituitrin, but after 1.0 mU there was regularly a distinct rise. This rise occurred during the increased electrolyte excretion, but its maximum did not necessarily coincide with the maximum of the sodium or potassium excretion.

In the experiments in which no antidiuresis ensued the mineral excretion showed no characteristic changes.

Pitressin. — The results obtained by using pitressin were essentially similar to those obtained with pituitrin. Three of six experiments with 0.25 mU gave a distinct antidiuretic response, whereas in four experiments with 1.0 mU an antidiuresis was again regularly

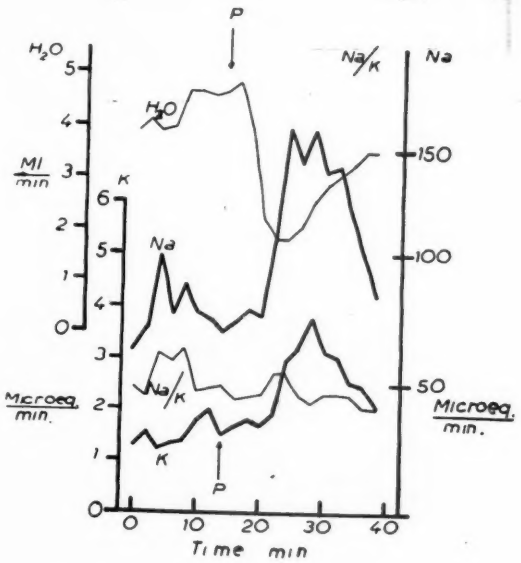

Fig. 1. — The results of an experiment made on dog D. At the arrow P, 0.25 milliunits of pituitrin was injected intravenously. Simultaneously with the antidiuretic response, the excretion of sodium and potassium rises, without any concomitant change in the ratio Na/K.

observed. The results of the experiments with an antidiuretic response are presented in Table I, and a typical example is shown in Fig. 2.

Pitocin. — An injection of 0.25 or 1.0 mU of pitocin, which had no effect on the urine flow, did not produce any characteristic changes in the sodium and potassium excretion. In all, five such experiments (three with 0.25 mU, two with 1.0 mU) were performed, with similar results.

No difference in the response to the postpituitary hormone or its fractions could be observed in the mineral excretion between dogs

TABLE 1

SUMMARIZING PRESENTATION OF THE EFFECTS OF 0.25 mU AND 1.0 mU OF PITUITRIN OR PITRESSIN ON URINARY SODIUM AND POTASSIUM EXCRETION IN EXPERIMENTS WITH DISTINCT ANTIDIURETIC RESPONSE. Na,K: + + = ABSOLUTE INCREASE; + = RELATIVE INCREASE; ± = NO CHANGE.

Na/K: + = INCREASE; ± = NO CHANGE.

NaCl = PREHYDRATION WITH 0.45 PER CENT NaCl; H_2O = PREHYDRATION WITH WATER.

DOGS J AND D				
Experiment	Dose mU	Na	K	Na/K
Pituitrin				
J—H_2O	0.25	++	+	+
J—NaCl	0.25	++	++	±
D—H_2O	0.25	++	++	±
D—H_2O	0.25	++	++	±
D—NaCl	0.25	+	++	+
J—H_2O	1.0	+	+	+
J—NaCl	1.0	++	+	+
D—H_2O	1.0	++	++	+
D—NaCl	1.0	+	±	+
Pitressin				
J—H_2O	0.25	++	+	±
J—NaCl	0.25	++	±	+
D—NaCl	0.25	++	++	±
J—H_2O	1.0	++	±	+
J—NaCl	1.0	++	++	+
D—H_2O	1.0	++	±	+
D—NaCl	1.0	++	++	+

prehydrated with tap water or 0.45 per cent NaCl. Neither did wide variations in the level of the sodium or potassium excretion affect the type of the results.

DISCUSSION

The present experiments demonstrate that even small, physiological doses of the postpituitary hormone are able to effect an increase in the sodium and potassium excretion in the dog. This is in an agreement with the results of Sartorius & Roberts. Moreover, the present results bring out that the actions of the posterior pitui-

tary hormone on the urine flow and on the mineral excretion have approximately the same threshold. In interpreting discrepant observations, differences in the experimental animals, in dosage and in the length of the sampling periods must be considered. Moderate doses of the postpituitary hormone cause an intense and prolonged antidiuresis, with a concomitant suppression of the elec-

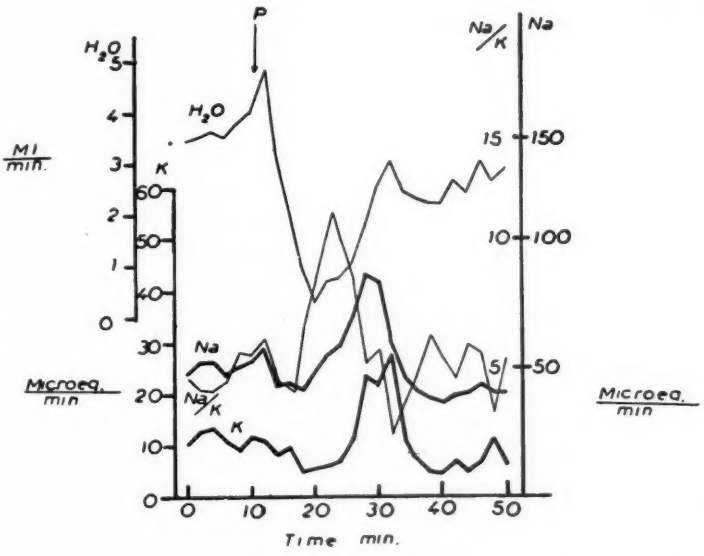

Fig. 2. — The results of an experiment made on dog J. At the arrow P, 1.0 milliunit of pitressin was injected intravenously. When the antidiuretic response passes its maximum, the excretion of sodium and potassium increases. The ratio Na/K shows a marked rise during the antidiuretic response.

trolyte excretion (Chalmers et al., Friberg, Karvonen, & Leppänen), whereas with higher doses the chloruretic action again becomes manifest. The analysis of samples collected over longer periods may also prevent the observation of small and rapidly passing changes.

It appears further that the factor responsible for the increase of the mineral excretion is either identical to or carried with the pressor-antidiuretic factor, as separated according to the conventional methods. This finding is in an agreement with those of Anslow et al., Sartorius & Roberts, and Roemmelt et al., but contrary to the results of Hare et al., similarly on dog.

The observed more or less parallel behaviour of sodium and potassium in these experiments suggests that the increase of the mineral excretion does not affect either of these ions specifically. The action of the postpituitary hormone differs in this respect in principle from that of the adrenocortical hormones which affect the sodium and potassium excretion in an opposite sense. This difference suggests that the action of the postpituitary hormone on the mineral excretion may lay primarily in an 'active' suppression of the reabsorption of the chloride ion, and that the observed changes in sodium and potassium excretion are only secondary, 'passive' in the physiological sense of the word. However, the chloride absorption hypothesis needs further validation. An increase in the glomerular filtration rate would effect a similar change. In the present results, a definite rise of the ratio Na/K occurred regularly after 1.0 mU of pituitrin or pitressin. It remains for further work to show whether changes in the ratio Na/K are compatible with the above hypothesis or whether they are due to a real relative selectivity in the reabsorption of these two ions.

In a few experiments, the results showed some differences from the typical pattern. This may depend on independent progressive changes in the excretion of either sodium or potassium, which changes may have covered the effects of postpituitary hormone.

SUMMARY

1. The effect of small doses of the postpituitary extract and its fractions on the excretion of Na, K, and water in dog has been observed.

2. The results demonstrate that pituitrin and pitressin effect, even in physiological doses causing antidiuresis, a simultaneous increase in sodium and, in a minor degree, in potassium excretion. Pitocin is without similar effects.

3. It is suggested that the postpituitary hormone does not affect either of these ions specifically and differs in that respect from the adrenocortical hormones. This difference may further suggest that the postpituitary hormone acts primarily on the chloride reabsorption.

REFERENCES

1. Anslow, W. P., Wesson, L. G., Bolomey, A. A., & Taylor, J. G.: Federation Proc. 1948:*7:*3.
2. Barclay, J. A., & Cooke, W. T.: XVIII Internat. Physiol. Congr. 1950:86.
3. Chalmer, T. M., Lewis, A. A., & Pawan, G. L. S.: J. Physiol. 1951: *112:*238.
4. Dicker, S. E., & Heller, H.: J. Physiol. 1946:*104:*353.
5. Fraser, A. M.: J. Pharmacol. 1937:*60:*89.
6. Fraser, A. M.: J. Physiol. 1942:*101:*236.
7. Friberg, O., Karvonen, M. J., & Leppänen, V.: Ann. Med. Exp. Biol. Fenn. 1950:*28:*144.
8. Hare, R. S., Hare, K., & Phillips, D. M.: Amer. J. Physiol. 1943:*140:* 334.
9. Hart, P. D., & Verney, E. B.: Clin. Sci. 1934:*1:*364.
10. Heller, H., & Stephenson, R. P.: Nature 1950:*165:*189.
11. Karvonen, M. J., & Laamanen, A. (1952) Unpublished.
12. Kuschinsky, G., & Bundschuh, H. E.: Arch. f. exper. Path. u. Pharmakol. 1939:*192:*683.
13. Leppänen, V., Forsander, O., & Mettinen, T. (1952) Unpublished.
14. McQuarrie, I., Manchester, R. C., & Husted, C.: Am. J. Dis. Child. 1932:*43:*1519.
15. Roemmelt, J. C., Sartorius, O. W., & Pitts, R. F.: Amer. J. Physiol. 1949:*159:*124.
16. Sartorius, O. W., & Roberts, K.: Endocrinology 1949:*45:*273.
17. Schaumann, O.: Experientia 1949:*5:*360.
18. Silvette, H.: Amer. J. Physiol. 1940:*128:*747.
19. Starling, E. H., & Verney, E. P.: Proc. Roy. Soc. B. 1925:*97:*321.
20. White, H. L., & Findley, T. Jr.: J. Clin. Investigation 1939:*18:*377.

FROM THE DEPARTMENT OF FORENSIC MEDICINE, UNIVERSITY OF HELSINKI

A COMPARISON OF THE LINEAR MEASUREMENT AND NUCLEAR VOLUME METHODS IN ACTIVITY DETERMINATIONS OF THE THYROID GLAND

AN EXPERIMENTAL STUDY WITH GUINEA PIGS USING THYREOTROPIN, IODINE, AND THIOURACIL

By

OSMO KANNAS and PEKKA TALA

(Received for publication October 27, 1952)

Greatly varying procedures have been employed in the determination of the activity of the thyroid gland. Probably the most widely used method is that presented by Starr and Rawson (12) for measurement of the mean epithelial cell height of 200 follicles, using an ocular micrometer. Based on studies carried out by the freezing-drying method by De Robertis (2) on the intracellular colloid of the normal and activated thyroid gland of the rat, De Robertis and Del Conte (3) described a cytological colloid droplet method. Kippen and Loeb (6) and Cutting (1), among others, employed a method involving a count of the mitoses.

Lever (9, 10) published a method of determination of thyroid activity based on mathematical principles. The d/n, which is the inner diameter divided by the number of cells in median or nearly median sections of the follicle, was used as a criterion of the activity of the follicle. The d/n corresponds to the apical cell breadth multiplied by π.

The volume of the cell nucleus was used as the criterion of thyroid activity by Ludwig (11), in whose opinion the epithelial cell height gives only an indication of the activity of the gland and is not an accurate criterion. He holds that the nuclear volume gives

an exact and differentiated index for the activity of the thyroid gland.

Uotila (14) proved mathematically the accuracy of a method for determining the colloid content of the entire thyroid gland, expressed as percentages of volume and weight. In experiments with guinea pigs, Uotila and Friedgood (15, 16) compared the results with those obtained in tests based on metabolism and found their method practicable. On the basis of this method, Uotila and Kannas (17) developed a new histoquantitative procedure by which the percentages of epithelium, colloid and stroma in the thyroid gland can be determined in a quantitative manner.

In carrying out standardization assays of thyrotropin, Tala (13) employed this method for the study of the effect of thyrotropin and thyroxin on the morphology of the guinea pig thyroid. In selecting an index that would reflect the degree of activity of the thyroid gland he found that the proportion of stroma in the gland remained low and practically constant in the tests, and he therefore arrived at the conclusion that the percentage of epithelium (E%) forms the index of thyroid activity. In one of his series of experiments the results obtained in the determinations of E% were compared with those obtained from the same specimens by the method of cell height measurement. It was found that under the prevailing test conditions the two methods were approximately equal in accuracy.

In the experiments described below it was the purpose chiefly to compare the method of E% determination by linear measurements with the above mentioned method of determining the nuclear volume, in order to learn whether the results obtained by the two methods are similar or divergent, and which method should be considered best suited for determinations of the activity of the thyroid gland.

MATERIAL AND METHODS

The test animals used were young male guinea pigs of as equal body weight as possible. The animals were reared raised under standard conditions of light, heat and food. Those selected for the tests were placed in an isolated room with a window, at a temperature of 20° C $\pm$ 2° C. At this time the males and females were separated, and each animal was kept in its own cage. The diet consisted of hay, Swedish turnips and oats.

The tests were made with thyrotropic hormone (TSH), sodium iodide and propyl thiouracil. The TSH used was Ambinon (Nordiska Organon), one ampoule (1 ml) of which contains ca 100 Heyl-Laquer (HL) guinea pig units of TSH, according to the manufacture's statement. A dilution of ca 1 unit per ml was made in physiological saline (0.9 per cent). One ml of this dilution was given once daily into the back of each test animal by subcutaneous injection, since this method of application appeared to be the least strenuous on the guinea pigs.

The control animals were simultaneously given injections of 1 ml of physiological saline. All the animals were killed by decapitation, which was the most rapid method and the least harmful to the thyroid gland.

The sodium iodide and propyl thiouracil were given in the form of powder that was mixed with the daily food of the animals. The amount given daily was 0.00004 g of sodium iodide or 0.004 g of propyl thiouracil, both in 10.0 g of oat meal.

Both lobes of the gland were dissected out carefully, cleaned, weighed and fixed immediately to prevent postmortal changes. Boun's fluid was used for fixation and the time was 6 hours. After fixation the specimens were treated with rising concentrations of alcohol. Each thyroid lobe was embedded in paraffin, then cut longitudinally with the microtome into two halves (sections of 50 per cent), and 8 to 10 slices were cut at 6—8 μ from the median surfaces. Staining was by Koneff's modification (7) of Mallory's azan method, which was used by Tala (13) in his earlier experiments.

Measurements of the nuclei were made in the usual manner along the principles devised by Jakobj (5). A monocular microscope with an attached Zeiss-Abbe draughting apparatus was used for tracing the circumferences of the nuclei. Drawing of the same nucleus twice was precluded by the use of a cross-ruled table. Cells with two nuclei were omitted, and calottes, decomposing or atrophied nuclei were discarded. Only the homogeneously stained and approximately round (difference in the length of the diameters not over 16 per cent) nuclear rings in the center of the field of vision were drawn.

In similarity with the E% measurements, the nuclear measurements were made from sections stained according to Koneff's modification of Mallory's azan method, and the slices were ca 6 μ in

thickness, which is considered to be the most advisable thickness by Leistner (8), among others.

Many investigators, as for instance Ludwig (11) and Wilflingseder (18), consider the measurement of 50 nuclei adequate when the variations in size are small. This number was regarded as sufficient also in the present work.

In consideration of the nature of the work, and since the occurrence of several classes of nuclei was not anticipated, the nuclei are classified in the tables according to the length of the diameter. This diameter has been obtained by adding the length of the longest diameter and that of the diameter at right angles to it, and dividing the sum by 2.

Table 1 shows the distribution of the nuclei of the 10 control animals according to the length of the nuclear diameter, and table 2 those of the 6 groups of test animals treated in different manners.

TABLE 1

NUCLEAR DIAMETERS OF THYROID EPITHELIAL CELLS OF CONTROL ANIMALS

Nuclear Diameter Length (μ)	Animal No.									
	31	32	33	34	35	36	37	38	39	40
4.5			1							
4.75	2		0							
5.0	3	4	3	3	5			4	1	5
5.25	5	2	2	3	7	1	5	4	13	5
5.5	18	19	15	23	23	17	22	32	21	28
5.75	7	10	15	14	9	17	19	8	9	10
6.0	3	7	6	5	4	5	1	1	1	0
6.25	11	8	8	2	2	10	3	1	5	2
6.5	1									
	94 μ^3									

In the graphs the dotted line depicts the curve obtained for the nuclei of the control animals. It shows the percentage of each diameter length in the total number of 500 nuclei. The curves for the 6 test groups of 5 animals each have been drawn along the same principle, with the difference that the total number of nuclei in each group is 250.

Thus no division into classes has been made, but if class K_1 (Regelklasse) is calculated by Jakobj's method from all the nuclei

TABLE 2

NUCLEAR DIAMETERS OF THYROID EPITHELIAL CELLS OF TEST ANIMALS

Nuclear Diameter Length (μ)	Group 1					Group 2					Group 3					Group 4					Group 5					Group 6				
	No. 1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30
4.5					1																									
4.75					1																								1	
5.0		1			1		1		2		3	1																2	2	
5.25		3	1	3	5	3	2	2	5	2	3	4	1	4				2						5	6	1	2	2	3	3
5.5	2	10	7	4	14	15	16	15	11	15	12	10	9	11	17		3	6	6	5	4	6	7	11	7	3	5	12	21	11
5.75	6	7	14	8	10	10	13	15	12	12	11	7	11	9	9	1	4	3	5	11	6	8	5	5	12	5	10	9	9	12
6.0	12	13	16	11	9	6	9	10	11	10	10	7	11	9	11	12	9	11	7	7	15	9	11	10	5	13	16	7	7	8
6.25	15	11	8	20	7	10	6	5	6	5	5	9	9	12	5	14	21	19	7	15	16	20	23	10	12	15	12	9	3	8
6.5	11	4	3	2	2	5	3	1	2	3	3	7	5	3	6	13	4	4	12	8	6	3	2	5	5	7	1	3	1	4
6.75	3	0	1	2		1		0	1	2	2	3	1	2	2	5	0	4	4	3	2	4	2	1	2	4	2	3	0	1
7.0	1	1						2		0	1	2	3			5	7	1	2	1	1			1	1	2	0	2	2	3
7.25										1							1		5					2			2	0	0	
7.5																	0		2									0	0	
7.75																	1											0	1	
8.0																												1		
	126	110	110	116	99	105	102	104	102	108	105	115	115	109	109	136	133	121	137	121	121	119	118	114	112	125	116	114	101	112
	112 μ^3					104 μ^3					111 μ^3					130 μ^3					117 μ^3					113 μ^3				

measured, it will fall on the diameter 5.90 μ, class $K_{1/2}$ on diameter 4.68 μ, and K_2 on diameter 7.43 μ. This corresponds to the following nuclear volumes: $K_{1/2} = 54$, $K_1 = 108$, and $K_2 = 215$. In order to obtain single figures for the nuclear volume for comparison with the E%, the nuclear volume for each animal and the average volume for each group were calculated. These figures are also given in the tables.

The linear measurements for calculation of the E% were performed by the histoquantitative method described by Uotila and Kannas (17) for determination of the percentages of the different components of the thyroid gland. The sections cut from the median surfaces of the gland were mounted on slides and placed in an apparatus by which the image was projected in powerful magnification downward onto a white plane by which the investigator was seated. This microprojector was constructed by Oy. G. W. Berg, Helsinki, and the measurements were carried out in the manner described by Tala (13).

The weight of the thyroid gland was determined after careful dissection and cleaning but before fixation, each lobe being weighed separately on a scale accurate to 0.5 mg.

RESULTS

The test animals were divided into 6 groups of 5 guinea pigs each. Ten animals served as controls.

The animals in groups 1, 2 and 3 were given subcutaneous injections of thyrotropin during 5 days (1 HL unit daily). After this the animals in group 1 were given propyl thiouracil in their food during 5 days (0.004 g daily), those in group 2 sodium iodide during the same length of time (0.00004 g daily), and those in group 3 the usual food with no addition. All the animals were killed on the eleventh day.

In groups 4, 5 and 6 the animals likewise received subcutaneous injections of thyrotropin for 5 days (1 HL unit daily). Simultaneously with this treatment the guinea pigs in group 5 were given in their food sodium iodide (0.00004 g daily) and the animals in group 6 propyl thiouracil (0.004 g daily). All the animals were decapitated on the sixth day.

TABLE 3. VALUES OBTAINED BY LINEAR MEASUREMENT METHOD AND NUCLEAR VOLUME MEASUREMENT METHOD

No.	Body Weight		Thyroid Weight				$10^5 \times$ Relative Weight		E %		C %	S %	Mean Nuclear Vol. (μ^3)		Treatment of Test Animals
	Initial	Final	Right L.	Left L.	Total	Average	Individual	Average	Indiv.	Ave.			Indiv.	Ave.	
1	235	210	23	26	49		23 ½		78		21	1	126		Group 1.
2	260	230	16	23 ½	39 ½		17		78		20	2	110		1 HL unit of TSH
3	260	230	20	30	50	43.1	21 ½	18.7	84	80	14	2	110	112	daily on 5 days, then
4	290	260	17	22 ½	39 ½		15		82		18	0	116		0.004 g propyl thiou-
5	245	230	17	20 ½	37 ½		16 ½		77		20	3	99		racil on 5 days
6	270	250	21	25 ½	46 ½		18 ½		75		22	3	115		Group 2.
7	210	180	15	15	30		16 ½		79		19	2	102		1 HL unit of TSH
8	250	230	13	16	29	33.7	12 ½	15.2	71	76	28	1	104	104	daily on 5 days, then
9	245	220	14 ½	17 ½	32		14 ½		74		25	1	102		0.00004 g sodium
10	240	220	15	16	31		14		81		17	2	108		iodide daily on 5 days
11	300	330	15	27	42		12 ½		75		24	1	105		Group 3.
12	310	335	28	30	58		17 ½		85		13	2	115		1 HL unit of TSH
13	280	310	23	24 ½	47 ½	46.3	15 ½	13.9	70	80	28	2	115	111	daily on 5 days, then
14	330	360	15	22 ½	37 ½		10 ½		85		14	1	109		ordinary diet on 5
15	310	350	18 ½	28	46 ½		13 ½		82		15	3	109		days
16	240	240	17	23 ½	40 ½		17		93		6	1	136		Group 4.
17	310	270	25	25	50		18 ½		83		16	1	133		1 HL unit of TSH
18	200	200	20	23	43	42.4	21 ½	17.9	87	87	11	2	121	130	daily on 5 days
19	280	270	20	18 ½	38 ½		14 ½		83		16	1	137		
20	230	220	21	19	40		18		89		11	0	121		
21	260	220	18 ½	26	44 ½		20		79		20	1	121		Group 5.
22	200	165	18	15	33		20		86		13	1	119		1 HL unit of TSH
23	220	185	14	16 ½	30 ½	38.1	16 ½	20.3	80	79	20	0	118	117	and 0.00004 g sodi-
24	240	175	17	17	34		19 ½		81		18	1	114		um iodide daily on 5
25	200	195	23 ½	25	48 ½		25		67		32	1	112		days
26	185	170	25	24 ½	49 ½		29		84		15	1	125		Group 6.
27	230	200	27	29	56		28		86		13	1	116		1 HL unit of TSH
28	230	210	21 ½	20	41 ½	45.4	20	23.5	71	81	27	2	114	113	and 0.004 g propyl
29	230	200	19	23	42		21		80		19	1	101		thiouracil daily on 5
30	230	195	16	22	38		19 ½		87		13	0	112		days
31–40		133	10.8	12.2		23	17 ½			67.8	31.1	1.1		94	Control Group. 10 animals; physiolo- gical saline on 5 days

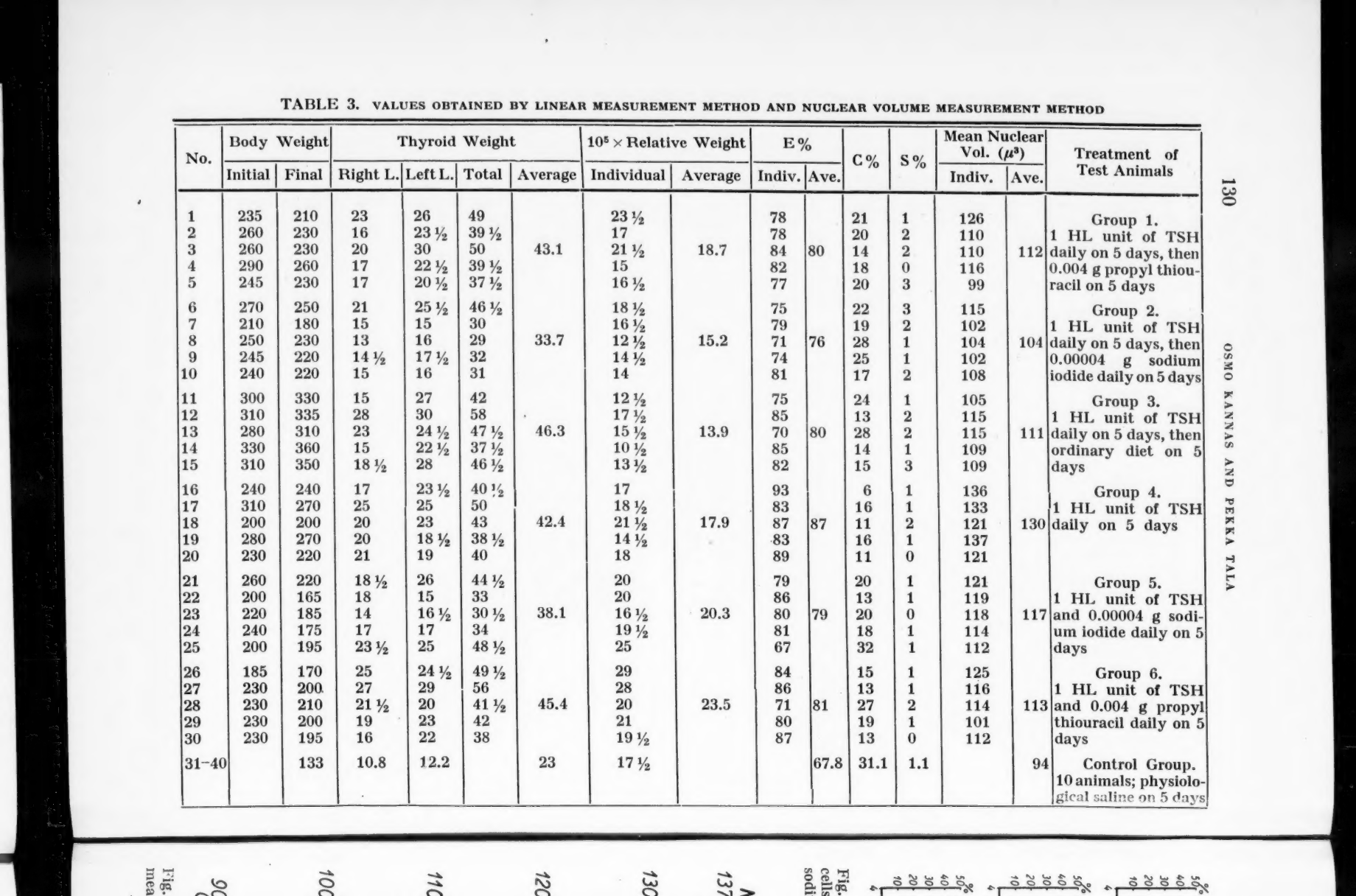

Fig. 1. Frequency curves of nuclear volumes in the follicular cells of the thyroid gland of rats. Group 1: 1 HL unit of TSH and 0.004 g propyl thiouracil daily on 5 days. Group 2: 1 HL unit of TSH and 0.00004 g sodium iodide daily on 5 days. Group 3: 1 HL unit of TSH daily on 5 days, then ordinary diet on 5 days. Group 4: 1 HL unit of TSH daily on 5 days. Group 5: 1 HL unit of TSH and 0.00004 g sodium iodide daily on 5 days. Group 6: 1 HL unit of TSH and 0.004 g propyl thiouracil daily on 5 days. Control group: 10 animals; physiological saline on 5 days.

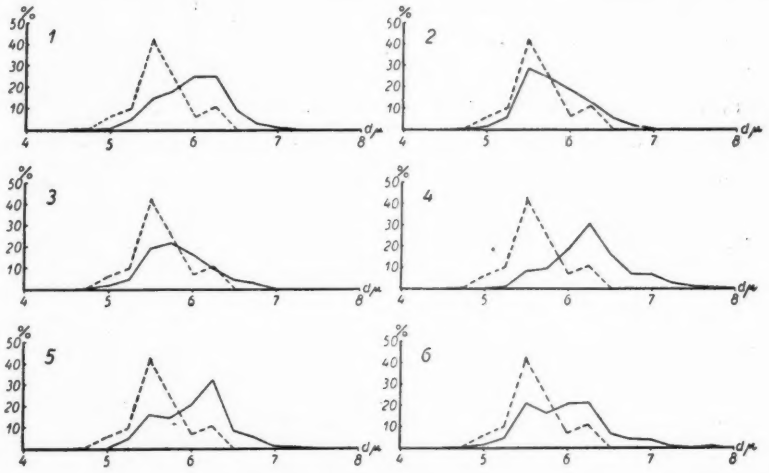

Fig. 1. — Percentile distribution of nuclear diameters of the thyroid epithelial cells in 6 groups of guinea pigs treated in different manners with thyrotropin, sodium iodide and propyl thiouracil. $d\mu$ = length of diameter in μ. Dotted line = control animals. Straight line = treated animals.

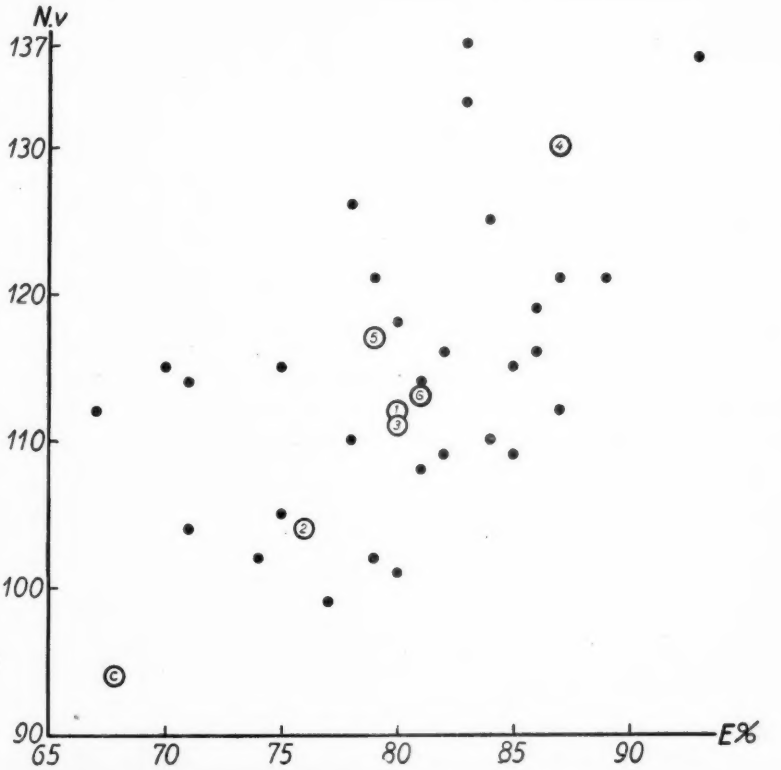

Fig. 2. — Graph showing comparison of the results obtained by the linear measurement method and the nuclear volume method. N.v. = nuclear volume. E % = percentage of epithelium.

The controls were given physiological saline for 5 days and killed on the sixth day.

The results obtained are shown in table 3 and figs. 1 and 2. For comparison of the linear measurement method with the nuclear volume method, the E% on the abscissa was plotted against the nuclear volume on the ordinate. The values for each animal are shown by a dot and the group averages by a circle. As will be seen from this graph, the averages fall on a nearly straight line, which goes to prove that the results obtained by the two methods are uniform.

DISCUSSION

The values obtained in the groups of guinea pigs treated in different ways were found to be almost the same whether calculated by the linear measurement method of E% determination or by the method of nuclear volume determination. Therefore, since the results obtainable by the two methods correspond, the advantage of one method over the other will be determined by its objectivity, speed and facility.

When the linear measurement method is used, an exact picture of the proportion of epithelium in relation to the other tissue components can be obtained already from a given cross section of the thyroid gland, since the lines of measurement extend nearly through the gland. The position of the straight lines across the sections is always the same and cannot be changed arbitrarily. In the nuclear measurements, on the other hand, the size of the nucleus indicates the activity of individual cell only. Therefore, in order to obtain an accurate picture of the thyroid activity within a given area it would be necessary to measure a vast number of nuclei. If only a small number is measured, a certain amount of selection unavoidably takes place and the measurements may possibly be made from an area of greater or lesser activity. Thus it will not be possible to obtain an exact indication of the activity of the thyroid gland.

In addition to possessing an advantage in this respect, the linear measurement of the epithelial percentage requires considerably less time to carry out. A study made in this respect in connection with the present work showed that nuclear measurement consumed at least three times as much time as linear measurement.

The tediousness and difficulty of the nuclear measurement method is also indicated by the fact that it requires a considerably greater number of mathematical calculations than the determination of the E%.

SUMMARY

Comparative calculations were made by two methods used in the determination of thyroid activity, i. e., the linear measurement method of epithelial percentage determination, and the method of nuclear volume calculation. The test animals were young male guinea pigs that were treated in different manners with thyrotropin, iodine and propyl thiouracil. In connection with the nuclear class determinations, the average nuclear volume of groups of animals was employed. In the opinion of the authors this gives clarity to the graphic curves and offers a better means of comparison with the average E% values.

The results obtained by the two methods were found to be approximately analogous. However, the linear measurement method of determining the percentage of epithelium in the thyroid gland is considered by the authors to be preferable in view of its objectivity, speed and facility.

REFERENCES

1. Cutting, W. C.: Endocrinology 1939:*25:*286.
2. De Robertis, E.: Am. J. Anat. 1941:*68:*317.
3. De Robertis, E., and Del Conte, E.: Rev. argent. de biol. 1944:*20:*88.
4. Heyl, J. G., and Laqueur, E.: Arch. internat. de pharmacodyn. et de thérap. 1934:*49:*338.
5. Jakobj, W.: Anat. Anz. 1931:*72:*236.
6. Kippen, A. A., and Loeb, L.: J. Pharmacol. & Exper. Therap. 1935: *54:*246.
7. Koneff, A. A.: Stain Technol. 1938:*13:*49.
8. Leistner, H.: Zeitschr. f. Zellforsch. u. mikr. Anat. 1936:*25:*34.
9. Lever, J.: Proc. Kon. Nederl. Acad. Wetens. 1948:*51:*1302.
10. Lever, J.: Onderzoekingen betreffende de Schildklierstructuur. Inaug. Diss., Utrecht, 1950.
11. Ludwig, K. S.: Acta Anat. 1950:*11:*146.
12. Starr, P., and Rawson, R. W.: Proc. Soc. Exper. Biol. & Med. 1937: *35:*603.

13. Tala, P.: Histoquantitative Studies on the Effect of Thyrotropin and Thyroxin on the Morphology of the Thyroid Gland, with Special Reference to Standardization of the Thyrotropic Hormone. Acad. Diss., Helsinki, 1952. Acta Endocrinol. 1952:*10:*Suppl. 9.
14. Uotila, U.: Acta Soc. Med. Fenn. Duodecim, 1940, Ser. A, Tom. XXIII, Fasc. 1.
15. Uotila, U., and Friedgood, H. B.: Acta Soc. Med. Fenn. Duodecim, 1940, Ser. A, Tom. XXIII, Fasc. 1.
16. Uotila, U., and Friedgood, H. B.: Ztschr. f.d. ges. exper. Med. 1943:*112:*579.
17. Uotila, U., and Kannas, O.: Acta Endocrinol. 1952:*11:*49.
18. Wilflingseder, P.: Forschungen und Forscher der Tiroler Ärzteschule 1945—47. Innsbruck, 1947, p. 343.

FROM THE DEPARTMENT OF PHARMACOLOGY, UNIVERSITY OF HELSINKI

PROCAINE

STUDIES ON ITS ABSORPTION AND ELIMINATION

by

LAURI ARO

(Received for publication November 5, 1952)

In recent years, several methods of operation have been introduced into surgical practice which require local anesthesia with considerably larger amounts of procaine than before. On the other hand, there are reports on cases in which procaine treatment has had a fatal issue. Considering all this, the present writer has studied, in animals and partly also in man, the absorption and hydrolytic decomposition of procaine, and also its acetylation and elimination in the organism. Particular efforts were made to find out clinically whether any toxic symptoms accompanying local anesthesia were ascribable to procaine and if so, in what measure they were dependent upon the size of the procaine fraction containing a free amino group (1).

CHEMICAL METHODS OF PROCAINE DETERMINATION

There are numerous methods of determining procaine from aqueous solutions and mixtures of drugs, but only few methods, however, applicable under biological conditions. Künster and Eissner (16), by a modified colorimetric method for drug mixtures, determined procaine from cerebrospinal fluid and blood and used β-naphthol for the formation of the azo dye. Willstaedt (25) determined procaine from muscle extract using aminonaphthyldisulphonic acid instead of β-naphthol. Dunlop (5) made the determination from dog blood and used β-naphthol for producing the azo

dye. Bullock and MacDonald (3) replaced β-naphthol by potassium sulphonate in determining procaine from the cerebrospinal fluid because they thought that in higher concentrations naphthol was not soluble enough. Koster, Shapiro, and Posen (15) proposed a method for the determination of procaine from cerebrospinal fluid in which vanillin, combining with weak sulphuric acid, formed a colour combination precipitated by potassium mercuric iodide and then dissolved. They believed that para-aminobenzoic acid could have no disturbing influence on the result because it did take part in this reaction. Kisch and Strauss (11) proposed a method for the determination of procaine and para-aminobenzoic acid from blood and urine. Brodie, Lief, and Poet (2) modified the Kisch and Strauss method by trying to obviate enzymatic hydrolysis in the blood sample by means of sodium arsenite; they carried out the extraction with ethylene chloride. Ting, Coon, and Conway (23) modified the method by using sodium fluoride and chloroform, respectively.

Since the introduction of sulphonamides into routine use a large number of methods have been proposed for their determination. These methods can also be used for procaine determination because the sulphonamide molecule contains a free amino group similar to that of procaine. Kinnunen (10), who made a comparative study of various methods of sulphonamide determination, found Kimmig's method (9) particularly useful.

The present writer used Kimmig's method for the determination of procaine. One difficulty of procaine determination is that since the NH_2 group of the acetylated fraction is liberated at acid hydrolysis when the substance from which the determination is made is boiled and since in this process trichloracetic acid readily decomposes, the *p*H readily rises, producing an error in photometric determination. In Kimmig's method, the *p*H remains constantly below one, which eliminates this source of error. It is chiefly for this reason, and partly because of post-war difficulties in acquiring reagents (the investigation was begun in 1946), that the writer found Kimmig's method useful.

The accuracy of the method was checked by determining extinction values from blood and urine to which a given amount of procaine had been added.

Successive determinations showed that the accuracy of the method for procaine was $\pm$ 5 per cent.

Since it could be assumed that the procaine fraction containing a free NH_2 group also contained some free para-aminobenzoic acid (Kisch-Strauss 1943), which, like procaine, is liable to acetylation,

the method was checked with respect to the quantitative liberation of the NH_2 group in acid hydrolysis by synthetic acetyl procaine and acetyl para-aminobenzoic acid.

HYDROLYTIC DECOMPOSITION

As an ester of para-aminobenzoic acid and diethylaminoethanol, procaine is decomposed into para-aminobenzoic acid and diethylaminoethanol at hydrolysis.

Goldenberg, Koster, and Warschaw (7) and other authorities who have carried out experiments *in vitro* with water solutions, find that the procaine molecule is rather easily hydrolysed when stored for a long time. It has also been found that hydrolysis readily takes place under the influence of temperature and, particularly, of acids and alkalis, and of catalysers.

Thieulin (22) extracted substances produced at the decomposition of procaine from rabbit urine. He identified procaine base, para-aminobenzoic acid, and diethylaminoethanol in crystals as results of procaine decomposition. Fosdick and Hansen (6) assumed that procaine, by decomposition, yields para-aminobenzoic acid and diethylaminoethanol. It is probable that the diethylaminoethanol decomposes further before its excretion into urine. Dunlop (5), who used dog blood, tried to isolate procaine by ether from para-aminobenzoic acid, which, he thought, was to some extent present in blood. Goldberg, Koster, and Warschaw (7) found that procaine was hydrolysed in blood *in vitro*. They conjectured that this was enzymatic hydrolysis. The most detailed study of the hydrolytic capacity of procaine in human serum is that of Kisch, Koster, and Strauss (13), who carried out experiments *in vitro* with large amounts of procaine. They found that the substance produced by hydrolysis was para-aminobenzoic acid and considered the hydrolysis to be enzymatic in nature, and gave the enzyme which they thought was responsible for the phenomenon the name »procaine esterase».

Goldberg, Koster, and Warschaw (7), who made a special study of procaine esterase, also came to the conclusion that the hydrolysis was enzymatic in nature, for the activity of the enzyme came to an end at the temperature of 56° C within three hours and the hydrolysis was inhibited by sodium fluoride. Hazard and Ravasse (1945) studied the hydrolytic capacity of serum and found that 100 γ of procaine in serum in the proportion of 1: 1 ml was completely hydrolysed at 37° C within 20 minutes. Citrated blood and old blood were hydrolysed more slowly and incompletely. Kisch, Koster, and Strauss (13) showed that in human serum hydrolysis comes to an end within 60 minutes at 56° C and that sodium fluoride strongly inhibited enzymatic activity. They found, further, that hydrolysis increased most between 45° and 50° C and that the amount of procaine hydrolysed was directly proportional to the length of the incubation period. The activity of the enzyme was greatest between

*p*H 6 and 8.6, and even large amounts of procaine, 1—0.06 mg/ml, did not alter the intensity of the hydrolysis. These authorities also found that in a mixture of procaine and para-aminobenzoic acid the intensity of the hydrolysis did not decrease and that hydrolysis increased by 30 per cent within 48 hours of the taking of the blood sample; then it remained unchanged for a few days, after which there was a slow decrease. They thought that procaine esterase was separate from acetylcholine esterase, tropine esterase, and lipase, but that procaine esterase was not specific to procaine only.

ACETYLATION IN THE ORGANISM

Acetylation, manifest in the capacity of the organism to combine CH_3CO with an amino group in aromatic amines or, occasionally, with a hydroxyl group, is one of the important general detoxication mechanisms. It has long been known, but has not received special attention until quite recently, after the introduction of sulphonamides into clinical routine. Acetylation inhibits their activity; the combinations thus formed may even be injurious to the organism as they tend to be precipitated in the urinary tract.

In the procaine molecule, as in the sulphonamides, the free amino group is in the benzole ring; thus, like the aromatic amino acids, procaine tends to acetylate in the organism. How far the subject of the acetylation is the procaine molecule itself it is not possible to say because the method of determination in which the NH_2 group is liberated in hydrolysis by hydrochloric acid causes the procaine molecule to disintegrate hydrolytically into para-aminobenzoic acid and diethylaminoethanol. On the other hand, para-aminobenzoic acid has long been known to be particularly liable to acetylation.

In the rabbit, paranitrobenzaldehyde is sectreted into urine as paranitrobenzoic acid and para-acetylaminobenzoic acid (4). Acetic acid and all substances contributing to the formation of acetic acid also contribute to the formation of acetylaminobenzoic acid in the rabbit (8). The rabbit and man offer particularly favourable ground for the acetylation of paraaminobenzoic acid (18, 19). It is generally recognised that acetate, pyruvate, ethyl alcohol, acetone, carbohydrates, and substances increasing carbohydrate metabolism, promote acetylation in the organism.

Among factors decreasing acetylation, Martin and Rennebaum (17) have emphasized the significance of vitamin B_1 or riboflavin deficiency.

According to Neubauer and Warburg (20) and Klein and Harris (14), acetylation takes place in the liver.

Goldenberg, Koster, and Warschaw (7) were the first to point out that by the side of procaine hydrolysis, some acetylation also takes place. They liberated the NH_2 group by means of hydrochloric acid and so proved that procaine was acetylated in the organism. They assumed that the process was enzymatic in nature because it was inhibited at 56° C within a few hours and they found that acetylation *in vitro* was slower that hydro-

lysis, but thought that in the organism it might be even rapid. Kisch, Koster, and Strauss (13) found that acetylation did not take place in the human organism if the blood cells were removed. Vandelli and Scaltriti (24) noticed that some acetylation took place in the heart and the kidneys. They also showed that in blood the acetylation of procaine came to an end within three hours of the administration of the drug and assumed that the occasional toxicity of procaine was due to the defective ability of the patient to acetylate this drug.

Terp (21) studied the acetylation of procaine in the rabbit, guinea pig, and dog. He found that acetylation took place in the rabbit and guinea pig, but not in the dog. In the liver of the rabbit, procaine hydrolysis and acetylation were rapid, an equilibrium being regained when ca. 60—80 per cent of the procaine was hydrolysed and ca. 50—80 per cent of the para-aminobenzoic acid acetylated.

OTHER MECHANISMS OF ELIMINATION

To judge from the well-known benzoic acid reaction, in which its carboxyl group combines with glycine and hippuric acid is produced, it seems possible that para-aminobenzoic acid also reacts to glycine to produce para-aminobenzoic glycine or para-amino hippuric acid. Further, considering the reaction mechanism peculiar to benzoic acid, it is natural to think that the carboxyl group of para-aminobenzoic acid combines with the OH group of glycuronic acid to form an ester called para-aminobenzoic glycuronate. A reaction of this type constitutes a common mechanism of detoxification in the organism. Reaction with glycine seems possible, too. — The organism is capable of adding an OH-group to the benzol ring, which may result in conjugation with glycuronic acid to produce compounds similar to glucosides.

One of the two products of the hydrolysis of procaine is an alcohol, diethylaminaethanol. Until quite recent times, this substance was considered pharmacologically ineffective. The organism possesses various mechanisms for its detoxification. It has been found that only one-third of the amount administered passes into the urine. The rest is evidently subjected to changes in the organism. The substance has been little studied.

ABSORPTION AND ELIMINATION OF PROCAINE IN SHEEP

The tests were made on three sheep approximately two years old. The quantity of blood needed for Kimmig's method (4 ml) is comparatively large. Since one test included several determinations, considerable amounts of blood, up to 70—100 ml, were required for each series. For this reason, the sheep is well suited for a test animal; besides, its capacity for acetylating procaine is

comparable with that of man. Many animals, such as the dog, do not acetylate procaine at all and cannot be used, therefore, for procaine determination (7).

Since highly uniform conditions are requisite for an adequate study of procaine absorption, 1 g of 1 per cent procaine solution was used in all the tests and 0.5 ml of 1: 1000 epinephrine in the control tests. The samples were taken, mainly, within two hours of the injection because most of the procaine was absorbed during

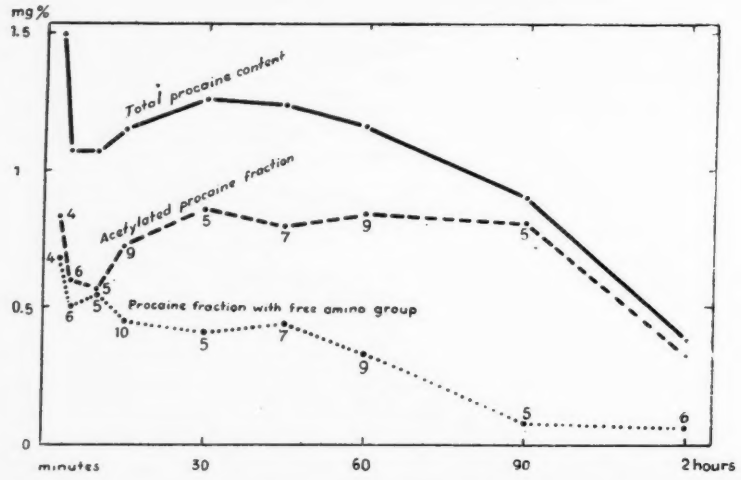

Fig. 1. — The procaine fractions (mg per 100 ml) of sheep blood without epinephrine influence, after the injection of one g of procaine intramuscularly. The numbers on the curves show the number of tests carried out, the calculated means of which are illustrated by the curves.

this time. Procaine was administered both with and without epinephrine, and determinations were made at given intervals on the free procaine and acetylated procaine fractions, which together were taken to represent the total procaine level of the blood. The values obtained at these intervals were added up; the means are presented in Figs. 1 and 2. Fig. 1 shows the procaine content of the blood following intramuscular injection of procaine without epinephrine and Fig. 2 the procaine content after injection with epinephrine.

The absorption of procaine injected into the sheep without epinephrine was rapid. The procaine content of the blood was 1.5 mg per 100 ml after a few minutes and remained at 1.1—1.2 mg

per 100 ml for about an hour. Under the influence of epinephrine the absorption of procaine was considerably slower, the total procaine content of the blood being at first less than 10 per cent of that following injection without epinephrine. After that, the procaine level rose steadily, but it took nearly an hour before it was nearly as high as the initial level of procaine injected without epinephrine.

The free procaine fraction was large even at the beginning, i.e., 0.7 mg per 100 ml, after which it decreased slowly and almost

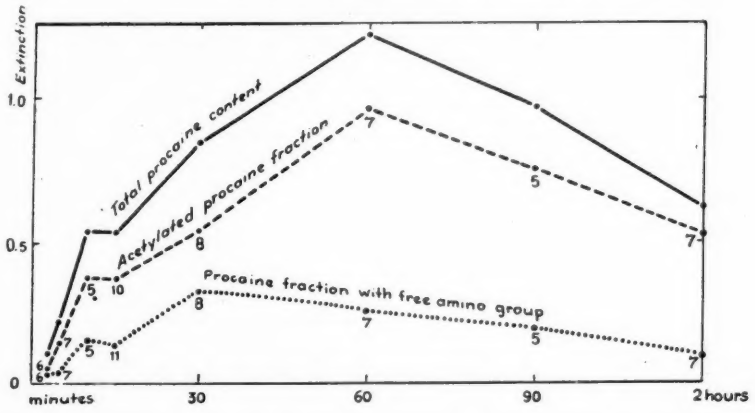

Fig. 2. — The procaine fractions (mg per 100 ml) of sheep blood under epinephrine influence, after intramuscular injection of one g of procaine. The numbers along the curves show the number of tests carried out, the calculated means of which are illustrated by the curves.

reached the zero point within two hours. Under epinephrine influence the free procaine fraction was largest 30 minutes after the administration of the drug, to decrease then slowly for two hours. The maximal amount was, however, only ca. 30 per cent of the maximal amount of procaine injected without epinephrine.

After the injection of the drug without epinephrine influence, the acylated procaine fraction was at first less than 50 per cent of the total procaine content, but after the first 10 minutes it rose steadily for two hours to ca. 90 per cent. Under the influence of epinephrine the acetylated procaine fraction was ca 60 per cent at first and increased then steadily to 75—90 per cent.

It is evident, thus, that epinephrine has a remarkable retarding effect on procaine absorption, and that it also prevents the maximal

free procaine content of the blood from being more than 30 per cent of that achieved without the influence of epinephrine. One consequence of the retarding influence of epinephrine seems to be that it facilitates procaine acetylation to judge from the fact that the fraction of acetylated procaine in blood was ca. 60 per cent at the very beginning and after a few minutes no less than 75—90 per cent of the total procaine content.

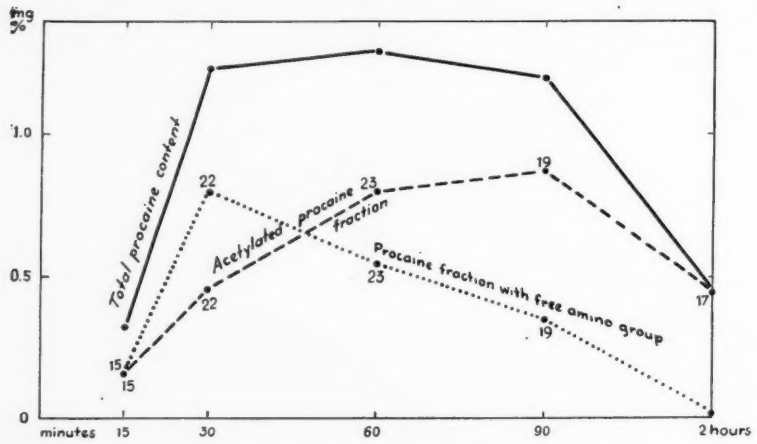

Fig. 3. — The procaine fractions (mg per 100 ml) of human blood under epinephrine influence in the first group, in which more than 20 mg of procaine per kg of body weight was injected intramuscularly. The numbers on the curves indicate the number of tests carried out, the calculated means of which are illustrated by the curves.

It is, thus, a peculiar characteristic of sheep blood that acetylation of procaine is extensive and rapid, so that in no more than three minutes ca. 50 per cent of the drug is acetylated even without epinephrine influence.

OCCURRENCE OF PROCAINE IN HUMAN BLOOD UNDER ANESTHESIA

On the basis of these animal experiments, determinations of the free and acetylated procaine fractions were made from the blood of surgically-treated patients anesthetised by 0.5 per cent procaine with epinephrine (1: 1000), with a maximum of 0.5 mg of epi-

nephrine per total dose of procaine. The majority of the patients were anesthetized by infiltration.

The determinations were carried out mainly at thoracic operations, such as thoracoplasty, which require anesthesia with large doses of procaine, but a number of determinations were also made at thyroidectomy and other minor operative measures. The series studied consisted of 71 cases collected over a period of ca. two years. Fig. 4 shows the procaine curves of those patients who received procaine more than 10 mg per kg of body weight, but it

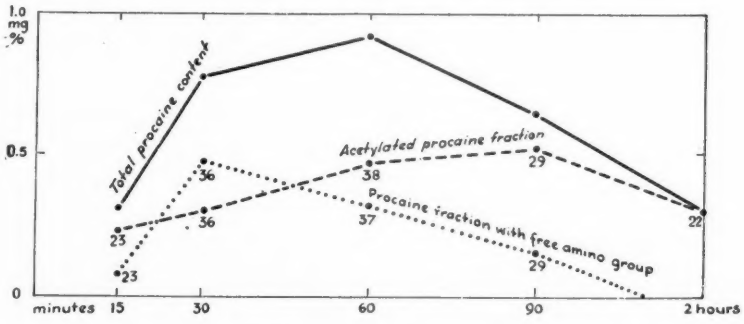

Fig. 4. — The procaine fractions (mg per 100 ml) of human blood under epinephrine influence in the first and intermediate groups, in which more than 10 mg of procaine per kg of body weight was injected intramuscularly. The numbers on the curves indicate the number of tests carried out, the calculated means of which are illustrated by the curves.

was not possible to draw curves for those patients who received less than 10 mg/kg. The determinations were carried out mainly 15, 30, 60, 90, and 120 minutes after the injection of procaine. The majority of those patients (Fig. 3) who received more procaine than 20 mg per kg of body weight (23 cases) underwent thoracoplasty, the largest dose being 64.5 mg per kg of body weight or altogether 4 g. In all the cases studied the procaine was administered as a single dose.

Untoward symptoms were present in 5 cases, all treated with 23.8—64.5 mg/kg. Vomiting and tachycardia were present in all these cases and sweating in two. The symptoms made their appearance 10—30 minutes after the injection of the drug. In the rest of the cases no toxic symptoms were noted except slight tachycardia,

which occurred frequently at the initial stage of anesthesia. When this symptom alone was present, it was, however, ascribed to epinephrine and therefore left out of consideration.

As seen from Fig. 00, the absorption of procaine was rapid, so that 30 minutes after the injection the quantity of the drug in the blood had reached its maximum. The most remarkable rise took place between 15 and 30 minutes of the injection. After that, the procaine level in the blood remained unchanged for approximately an hour and then began to fall slowly.

In the free procaine level an abrupt rise was also noticeable during the second quarter of an hour, but after 30 minutes there was a clear fall, and in two hours free procaine had disappeared completely from the blood stream.

The acetylated procaine fraction was 60 per cent of the total procaine content of the blood 15 minutes after the injection, after which there was some decrease as the procaine was rapidly absorbed, so that at 30 minutes it was only ca. 40 per cent. During the second 30 minutes, however, the procaine fraction increased to ca. 60 per cent, and during the second hour to 100 per cent. Man, thus, possesses a remarkable ability to acetylate procaine.

It was shown, in the foregoing, that the free procaine fraction reached its maximum within the first 30 minutes of the injection; it was at this stage that the general effect of procaine was most distinct. Five patients showed toxic symptoms in the form of vomiting, tachycardia, and sweating 30 minutes after the injection of the anesthetic. In these cases 23.8—64.5 mg of procaine were used per kg of body weight, the free procaine content of the blood ranging from 1.4 to 2.4 mg per 100 ml. The possibility of procaine intoxication is obviously to be taken into consideration in these cases.

EXCRETION OF PROCAINE INTO URINE

The free and acetylated procaine fractions of the urine were determined according to Kimmig's method. Urine determinations were carried out in 20 operatively-treated cases. The procaine was injected intramuscularly as a 0.5 per cent solution together with epinephrine. The urine samples were collected 10, 24, 48, and 72 hours after the injection.

In the series examined, 44.7—91.5 per cent (average 70.1 per cent) of the procaine administered was excreted into urine; 80.8—100 per cent of this underwent acetylation, the free procaine fraction being 0—19.2 per cent.

In the 17 cases in which the excretion could be studied by a uniform method, the following proportions of the procaine injected passed from the body with the urine:

0—10	hours	59	per cent
10—24	»	14.4	» »
24—48	»	2.6	» »
48—72	»	0	» »
48	hours	76.0	per cent

Most of the procaine administered to the patient was removed from the body in urine; before its excretion into urine, most of the drug underwent acetylation in the organism.

DISCUSSION

When procaine was injected without epinephrine, the procaine content of the blood reached its maximum only a few minutes after the injection. The retarding effect of epinephrine on procaine absorption was, however, evident. In man, when procaine was given in combination with epinephrine, and in the sheep, when epinephrine was given in addition to procaine, the procaine level of the blood was highest 60 minutes after the injection, after which it fell steadily, until the procaine disappeared entirely from the blood within two hours or so. Comparison between the acetylating capacity of man and that of sheep showed that 15 minutes after injection the acetylated procaine level was ca. 60 per cent in man and ca. 70 per cent in the sheep. At 30 minutes the figures were ca. 40 and ca. 80 per cent respectively; after that, there was hardly any difference at all, the percentage being 80—100. When the free procaine fraction reached its maximum, the acetylated procaine level was more than 80 per cent in the sheep, but only ca. 40 per cent in man. Even the sheep was given procaine without epinephrine, it was able to acetylate at least 50 per cent of the procaine administered. There was thus a distinct parallelism between man and

the sheep with respect to the absorption and elimination of procaine in man and those in the sheep. It is obvious, however, that the sheep had a greater capacity to acetylate procaine, to judge from the fact that when the free procaine content of the blood was greatest, man acetylated only about half the amount acetylated by the sheep.

When 0.5 mg of epinephrine was injected together with procaine, the blood procaine fraction containing a free amino group was greatest, both in man and the sheep, 30 minutes after the injection. The general effect of procaine could be assumed to most distinct at this stage; an attempt was made, therefore, to find out whether the patient would show any symptoms or signs of procaine intoxication. Part of the patients received considerable amounts of procaine; in 23 cases the amount administered exceeded 20 mg per kg of body weight, with a maximum dose of 64.5 mg per kg, the maximum procaine fraction which contained a free amino group being 2.4 mg per 100 ml. Five patients who received 23.2—64.5 mg of procaine per kg of body weight and in whom the procaine fraction containing a free amino group ranged from 1.4 to 2.4 mg per 100 ml had nausea, vomiting, tachycardia, and marked sweating 30 minutes after the injection of the drug. The symptoms were ascribed to mild procaine intoxication. It seems, therefore, that when administered together with epinephrine, procaine has not a particularly toxic action, to judge from the fact that untoward symptoms were present only after the injection of at least 23.2 mg of procaine per kg of body weight.

Goldenberg, Koster, and Warschaw (7) found that most of the procaine, 94—96 per cent, was excreted from the organism in acetylated form, while only 2 per cent of it was excreted as para-aminobenzoic acid and very little, 2—4 per cent, as free procaine. Brodie, Lief, and Poet (2) stated that ca. 90 per cent of the injected procaine is excreted, 2 per cent of which is excreted as free procaine and the rest in acetylated form.

The present writer found that most of the injected procaine, 70 per cent on an average, was secreted into the urine. Of this amount, 80.8—100 per cent was acetylated, the fraction containing a free amino group constituting 0—19.2 per cent of it.

SUMMARY

In the investigation here reported, the determination of both free and acetylated procaine was carried out by Kimmig's method, checked by hydrolysing of the acetylated compounds of procaine and para-aminobenzoic acid.

The absorption and elimination of procaine was studied in the sheep by means of determinations from the blood at short intervals over a period of two hours.

Similar determinations of the procaine content were carried out in 71 human subjects anesthetised by procaine. In all these cases, 0.5 mg epinephrine was given as well. Five patients showed, during the initial stage of anesthesia, untoward symptoms suggestive of procaine intoxication; these patients received 23.8—64.5 mg of procaine per kg of body weight, the free procaine fraction of the blood ranging from 1.4 to 2.4 mg per 100 ml.

An evident parallelism was noted between man and the sheep in the absorption and elimination of procaine, though it is obvious that the sheep is capable of acetylating procaine in a somewhat greater measure than man.

In man, most of the procaine administered was excreted into the urine. Most of the excreted procaine was acetylated, the fraction which contained a free amino group being small.

REFERENCES

1. Aro, L.: Suppl. XVIII Duodecim 1950.
2. Brodie, B. B., Lief, P. A., Poet, R.: J. Pharmacol. 1948:*94:*359.
3. Bullock, K., MacDonald, A.: J. Pharmacol. 1938:*62:*39.
4. Cohn, R.: Z. Physiol. Chem. 1893:*17:*274.
5. Dunlop, J. G.: J. Pharmacol. 1935:*55:*464.
6. Fosdick, L. S., Hansen, H. L.: Dent. Cosmos 1931:*73:*1082.
7. Goldberg, A., Koster, H., Warschaw, R.: Arch. Surg. 1943:*46:*49.
8. Hensel, M.: Z. physiol. Chem. 1915:*95:*401.
9. Kimmig, J.: Dtsch. med. Wschr. 1943:*69:*531.
10. Kinnunen, O.: Duodecim 1946:*62:*84.
11. Kisch, B. Strauss, E.: Exper. Med. a. Surg. 1943:*1:*66.
12. Kisch, B. Strauss, E.: Exper. Med. a. Surg. 1943:*1:*367.
13. Kisch, B., Koster, H., Strauss, E.: Exper. Med. a. Surg. 1943:*1:*51.
14. Klein, J. R.: Harris, J. S.: J. Biol. Chem. 1938:*124:*613.
15. Koster, H., Shapiro, A., Posen, E.: J. Labor. a: Clin. Med. 1936:*21:* 1096.

16. Künster, H., Eissner, W.: Münch. Med. Wschr. 1930:*77:*622.
17. Martin, G. J., Renne-Baum, E. H.: Biol. Chem. 1943:*151:*416.
18. Muenzen, J. B., Cerecedo, L. R., Sherwin, C. P.: J. Biol. Chem. 1925:*63:*XVI.
19. Muenzen, J. B., Cerecedo, L. R., Sherwin, C. P.: J. Biol. Chem. 1926:*67:*469.
20. Neubauer, O., Warburg, O.: Z. physiol. Chem. 1910:*70:*1.
21. Terp, P.: Acta Pharmacol. et Toxicol. 1951:*7:*381.
22. Thiebulin, R.: C.r. Soc. Biol. 1920:*83:*1347.
23. Ting, K. S., Coon, J. M., Conway, A. C.: J. Labor. a. Clin. Med. 1949:*34:*822.
24. Vandelli, I., Scaltriti, E.: Abstr. in Krbl. 1943:*116:*292.
25. Willstaedt, H.: Biochem. Z. 1934:*269:*182.

FROM THE DEPARTMENT OF SEROLOGY AND BACTERIOLOGY, UNIVERSITY OF HELSINKI

DETERMINATION OF BACTERIAL RESISTANCE FOR CLINICAL USE

by

W. J. KAIPAINEN

(Received for publication November 28, 1952)

The determination of bacterial sensitivity to antibiotics is an important factor in the effective use of the latter. The Oxford cup plate method (1), modified (2, 6, 8, 9, 10) in different ways, is still the most generally used by various laboratories. The greatest weakness of sensitivity determinations is that they indicate the sensitivity *in vitro*. Efforts have been made to imitate conditions *in vivo* by using dilution methods (4), with the antibiotic concentration of the culture medium corresponding to the known concentrations that a certain antibiotic has e.g. in blood after the so-called normal dosage. The interest of the present author was attracted by the relationship between plate technic and serial dilution assay.

The investigation was carried out by the serial dilution method alongside the routine sensitivity determinations. The culture medium employed in the serial dilution method was broth (pH 7.4). A bacterial suspension was prepared of 24-hour broth culture diluting it to 10^{-4} with sterile broth. A dilution series was made with this suspension, with three tubes for each antibiotic; the antibiotic concentrations were selected as follows:

1. The highest concentration level in blood caused by normal dosage.

2. The average concentration level in blood caused by normal dosage, (3. 5. 11).

3. A concentration equalling $^1/_4$ of the former (Item 2).

The concentrations in the tubes were thus:

	Tube No. 1	2	3
Penicillin	0.8 unit/ml	0.2 unit/ml	0.066 unit/ml
Dihydrostreptomycin	16 μg/ml	4 μg/ml	1 μg/ml
Aureomycin	12 »	3 »	0.75 »
Chloromycetin	20 »	5 »	1.25 »
Terramycin	12 »	3 »	0.75 »

In the plate assay method peptone-free blood agar was used as the culture medium, 15 ml poured onto each plate. A disk of filter paper, diameter 1.5 cm, was placed on the plate. 0.05 ml of antibiotic solution was dropped onto the disk. The concentrations of the various antibiotics were:

Penicillin	20 units/ml
Dihydrostreptomycin	500 μg/ml
Aureomycin	500 »
Chloromycetin	500 »
Terramycin	500 »

The disk was removed after it had been on the plate for 15 min. and 2 ml of a bacterial suspension in saline (dilution 10^{-4}) was pipetted onto the plate. The entire surface of the blood agar was moistened with the suspension and the surplus discarded, and the plate placed in an incubator for 24 hours, after which the reading was taken. The zone of inhibition was measured, and the following values were given to it:

Zone of Inhibition	*Sensitivity Value*
$<$ 10 mm	0
10—19 »	1
20—29 »	2
30—39 »	3
40—49 »	4
$>$ 50 »	5

The sensitivity of 137 micro-organisms was studied. The results are given in Tables 1—5. Table 1 shows that the two methods correspond to one another when penicillin is in question. It is typical that the bacterial strains are either sensitive or resistant, and

TABLE 1

COMPARISON OF BACTERIAL SENSITIVITY VALUES TO PENICILLIN OBTAINED BY THE DILUTION METHOD AND THE DISK PLATE METHOD

Growth-inhibiting Penicillin Concentration in Broth	Number of Strains	Corresponding Sensitivity Values on Blood Agar Plates (Number of Strains)					
		0	1	2	3	4	5
0.066 unit/ml	47			10	24	8	5
0.2 »	9	2	1	3	3		
0.8 »	4	3	1				
>0.8 »	76	73	1	2			

TABLE 2

COMPARISON OF BACTERIAL SENSITIVITY VALUES TO DIHYDROSTREPTOMYCIN OBTAINED BY THE DILUTION METHOD AND THE DISK PLATE METHOD

Growth-inhibiting Dihydrostreptomycin Concentration in Broth	Number of Strains	Corresponding Sensitivity Values on Blood Agar Plates (Number of Strains)					
		0	1	2	3	4	5
1 μg/ml	43			13	20	10	
4 »	32		1	10	20	1	
16 »	35	4	5	17	5	3	1
>16 »	28	16	6	2	4		

TABLE 3

COMPARISON OF BACTERIAL SENSITIVITY VALUES TO AUREOMYCIN OBTAINED BY THE DILUTION METHOD AND THE DISK PLATE METHOD

Growth-inhibition Aureomycin Concentration in Broth	Number of Strains	Corresponding Sensitivity Values on Blood Agar Plates (Number of Strains)					
		0	1	2	3	4	5
0.75 μg/ml	83	1	1	25	39	16	1
3 »	16		1	8	7		
12 »	21	10		11			
>12 »	18	14	4				

not many strains come in the so-called border area between sensitive and resistant. With regard to dihydrostreptomycin too, Table 2, both determination methods show a fair degree of accord, but several strains lie within this border area.

With aureomycin, Table 3, this area is somewhat larger with the plate method, and the serial dilution technic appears to give a

TABLE 4

COMPARISON OF BACTERIAL SENSITIVITY VALUES TO CHLOROMYCETIN OBTAINED BY THE DILUTION METHOD AND THE DISK PLATE METHOD

Growth-inhibition Chloromycetin Concentration in Broth	Number of Strains	Corresponding Sensitivity Values on Blood Agar Plates (Number of Strains)					
		0	1	2	3	4	5
1.25 μg/ml	74		1	13	44	15	1
5 »	29		1	12	16		
20 »	14	1	2	6	5		
>20 »	20	16	4				

TABLE 5

COMPARISON OF BACTERIAL SENSITIVITY VALUES TO TERRAMYCIN OBTAINED BY THE DILUTION METHOD AND THE DISK PLATE METHOD

Growth-inhibition Terramycin Concentration in Broth	Number of Strains	Corresponding Sensitivity Values on Blood Agar Plates (Number of Strains)					
		0	1	2	3	4	5
0.75 μg/ml	87		2	13	42	23	7
3 »	18		1	11	6		
12 »	14	3	5	4	2		
>12 »	18	17				1	

larger number of sensitive strains. The disk plate method, in fact, has been considered to give a slightly more unfavourable picture of the effectiveness of aureomycin. With chloromycetin, Table 4, the dilution method gives more sensitive strains than the plate method. This, partly at least, may be due to the fact that the concentrations in the dilution method are fairly high, corresponding to the blood concentrations observed. With terramycin also, Table 5, the dilution method gives somewhat more sensitive strains than the plate method.

It seems that the concentrations in the plate method are somewhat too low for aureomycin, chloromycetin and terramycin. However, no higher concentrations can be achieved because of the poor solubility of the commercial antibiotic preparations that have had to be used. In addition, antibiotic manufacturers today recommend a smaller dosage than earlier, making the aureomycin, chloromycetin and terramycin concentrations used with the dilution method somewhat too high.

If we assume that the clinicians know the antibotic concentrations present in the different organs in connection with the normal dosage, it would seem that the sensitivity results could well be expressed in micrograms/ml. However, as it is evidently difficult for the clinician to remember the possible concentrations, it can be concluded that the sensitivity of bacteria in vitro should be reported in some other way. If the following frequently observed indications of sensitivity are used:

resistant, very slightly sensitive, slightly sensitive, moderately sensitive, highly sensitive and very highly sensitive several degrees of sensitivity (very slightly, slightly and moderately) will coincide with the so-called border area. It is hardly necessary to provide clinicians with such detailed results as the results vary to some extent for technical reasons alone. We (7) adopted the following scale of indications in 1950:

1. *sensitive* — the bacterial growth is inhibited in vitro in an antibiotic concentration equal to the probable concentration in blood with customary dosage (so-called mean concentration).
2. *less sensitive* — an antibiotic concentration equal to the above-mentioned mean concentration does not inhibit bacterial growth in vitro, though the growth is inhibited by a concentration equal to the maximal antibiotic concentration in the blood.
3. *resistant* — a concentration equal to the above-mentioned maximal concentration in the blood does not inhibit bacterial growth in vitro.

In the paper disk method the values indicated previously correspond to the following degrees of sensitivity:

values 3—5 = sensitive
» 2 = less sensitive
» 0—1 = resistant

As the mode of action of antibiotics is different in vitro and in vivo it is evident that the above classification only gives a general indication. Data on the value of this method in clinical use will be published separately.

SUMMARY

1. The correlation between the plate assay and serial dilution method was studied on 137 bacterial strains with penicillin, dihydrostreptomycin, aureomycin, chloromycetin and terramycin. There was no significant difference between the two methods, though a slight disagreement was noticed if the sensitivity was tested against aureomycin, chloromycetin and terramycin.

2. In the author's opinion the following definitions of sensitivity are adequate for clinical use: sensitive, less sensitive and resistant.

REFERENCES

1. Abraham, E. P., Chain, E., Fletcher, C. M., Gardner, A. D., Heatley, N. G., Jennings, M. A., and Florey, H. W.: Lancet 1941: *241:*177.
2. Andina, F., and Allemann, O.: Therap. Umschau 1950:*7:*1.
3. Buggs, C. W., Pilling, M. A., Bronstein, B., and Hirschfeld, J. M.: J. clin. Invest. 1946:*25:*94.
4. Diding, N., and Wallmark, G.: Nord. Med. 1951:*46:*1191.
5. Frisk, A. R.: Nord. Med. 1950:*43:*323.
6. Hoyt, R. E., and Levine, M. G.: Science 1947:*106:*171.
7. Kaipainen, W. J., and Vartia, K. O.: Suomen Lääkärilehti 1951:*13:*1.
8. Lund, E., Funder-Schmidt, B., Christensen, H., and Dupont, A.: Acta Path. Microbiol. Scand. 1951:*29:*221.
9. Spaulding, E. H., and Anderson, T. G.: J.A.M.A. 1951:*147:*1336.
10. Vincent, J. G., and Vincent, H. W.: Proc. Soc. Exp. Biol., N. Y. 1944:*55:*162.
11. Welch, H.: Ann. N. Y. Acad. Sci. 1950:*53:*253.

FROM THE DEPARTMENT OF SEROLOGY AND BACTERIOLOGY AND THE SECOND MEDICAL CLINIC, UNIVERSITY OF HELSINKI

»JOKINEN'S SERUM PRECIPITATION TEST»

COMPARISON WITH ELECTROPHORETIC PROTEIN ANALYSIS

by

E. J. JOKINEN and W. J. KAIPAINEN

(Received for publication December 3, 1952)

One of the writers (E. J. Jokinen) has previously reported on a method (3, 4) which differentiates between the majority of the sera of rheumatic patients and the normal. Cortisone treatment changes the mode of reaction towards the normal. The probable cause of this phenomenon is perhaps linked to the serum proteins. This conception is supported by the fact that positive reactions may be found also in many non — rheumatic cases especially when changes in serum proteins are known to occur (5), and further by the fact that cortisone treatment has a tendency to normalise the serum proteins (2).

The purpose of the present investigation has been to study whether any correlation can be stated between »Jokinen's serum precipitation test» and electrophoretic analysis of protein.

TECHNIQUE

2.5 ml of 94 per cent ethyl alcohol was measured out into a test tube. 0.25 ml of the serum to be examined was pipetted into it at room temperature, a drop at a time, when a white precipitate formed. The test tube was put in the icebox for the night. It was then centrifuged at 2500 r.p.m. for 5 minutes. The supernatant was poured off and the test tube was allowed to stand, turned upside down, at room temperature for 15 minutes. Then 1 ml of 95 per cent

sulphuric acid was measured out into the test tube onto the precipitate. After 5 minutes at room temperature the result was read. It was considered positive (+) if the precipitate had dissolved completely and a strong brown colour was produced; slightly positive (±) if the precipitate had dissolved partly and some brown colour was produced; and negative (—) if the precipitate had not dissolved and the sulphuric acid remained colourless. The investigation was performed on two successive days, not less than four times in all for each sample, and the average of the results was taken as the final reading. The technique was that employed by one of the authors in his work on rheumatic diseases referred to above (4). For the sake of brevity, it will be called in the following the »serum precipitation test».

The electrophoretic protein analyses were effected with Antweiler's micro-electrophoresis apparatus (1). The buffer employed was Dole's veronal, veronal-sodium buffer: ionic strength 0.12, pH 8.6. Dialyzing time was 1—2 hours. One part of the dialyzed serum was diluted with 3 parts of the buffer. In one case, with a low total protein content, the dilution 1: 3 was used. The current was 1.8 mA, running time 18 min. Double determination was made of each serum, and the differences were always under 1.5%, the limit above which, according to Antweiler, a new analysis is required (1).

MATERIAL

The material comprised 20 sera taken from hospital patients. The distribution of diagnoses was as follows:

	Number of cases
Rheumatoid arthritis	5
Rheumatic fever	7
Osteo-arthritis	4
Sarcoidosis	1
Varicose veins	1
Carcinoma of the lung with bone and liver metastases	1
Chronic nephritis	1

RESULTS

The results are shown in the attached table, where the cases are arranged by the results of the »serum precipitation test». (All four readings are included in each case.)

TABLE

COMPARISON OF RESULTS OBTAINED IN 20 CASES BY »JOKINEN'S SERUM PRECIPITATION TEST» AND BY ELECTROPHORESIS.

Case N:o	»Jokinen's Serum Precipitation Test.» Final Reading.	Electrophoresis						
		Total Protein	Albumin per cent	Globulin per cent				Alb/Glob.
				α_1	α_2	β	γ	
1	—	6.80	68.09	1.18	6.03	5.15	19.65	2.13
2	—	7.58	55.54	2.77	10.16	14.78	16.75	1.18
3	—	7.40	51.35	1.35	8.24	17.97	21.08	1.06
4	±	6.84	50.58	4.82	14.04	10.23	20.32	1.02
5	±	7.56	59.66	5.29	13.23	7.28	14.55	1.48
6	±	7.82	53.45	2.94	7.67	15.86	20.08	1.15
7	±	7.11	49.23	5.63	9.14	18.28	17.72	0.97
8	±	8.40	36.19	6.19	12.86	15.12	29.64	0.57
9	±	8.24	49.03	3.40	9.22	12.14	26.21	0.96
10	±	8.07	43.87	12.02	22.30	9.54	12.27	0.78
11	±	6.14	32.57	9.45	26.06	9.77	22.15	0.48
12	±	8.50	45.65	4.35	17.88	11.76	20.35	0.84
13	±	7.98	50.13	5.26	5.01	16.92	16.04	1.01
14	±	8.38	50.84	5.73	8.00	15.87	19.57	1.03
15	+	7.16	54.05	6.00	14.66	8.80	19.23	1.18
16	+	7.14	39.78	12.04	14.99	15.69	17.51	0.66
17	+	7.48	32.62	6.95	17.65	5.88	36.10	0.45
18	+	8.40	29.17	1.07	7.62	18.10	44.05	0.41
19	+	5.29	47.64	7.94	17.01	11.91	15.50	0.91
20	+	8.45	45.44	2.37	16.21	13.96	22.01	0.83
Normal average (according to Antweiler)		Alb.	α			β	γ	Alb/glob.
		63.20	9.00			13.70	14.10	1.71

DISCUSSION

A comparison of the results of the »serum precipitation test» with the different peaks in the electrophoretic picture shows no absolute correlation. However the globulins as a rule have increased proportionately and the albumin correspondingly decreased in

cases that have reacted positively to the »serum precipitation test». Generally the more strongly positive the test the more evident were the changes in the electrophoretic picture. Most distinctly this may be observed on the globulin side with α_1, α_2 and γ fractions. As, on the other hand, healthy subjects are known (4) to react negatively, it seems possible that an abnormal albumin globulin ratio might be one of the reasons for a positive result. However on the basis of our material this does not always follow since there are also exceptions from the rule (cases 13, 14, 15). It is also possible, that the protein changes may not be the cause but only a parallel phenomenon.

SUMMARY

A comparison of results obtained by »Jokinen's serum precipitation test» with an electrophoretic protein analysis in 20 cases showed that in the positive-reacting sera the grobulins had generally increased and the albumin decreased.

REFERENCES

1. Antweiler, H. J.: Die quantitative Elektrophorese in der Medizin. Springer Verlag. Berlin 1952.
2. Jager, B. V., Brown, H., and Nickerson, M.: J. Lab. Clin. Med. 1951:*37:*431.
3. Jokinen, E. J.: Ann. Med. Exp. Biol. Fenn. 1952:*29:*78.
4. Jokinen, E. J.: Ann. Med. Exp. Biol. Fenn. (printing.)
5. Kagan, B. M.: Ann. J. Med. Sci. 1943:*206:*309.

FROM THE STATE SERUM INSTITUTE, HELSINKI, AND FROM THE DEPARTMENT OF VIRUS RESEARCH, UNIVERSITY OF HELSINKI

PURIFICATION AND PROPERTIES OF VIRUS HEMAGGLUTINATION INHIBITORS OBTAINED FROM EGGWHITE

by

KARI PENTTINEN[1] and VEIKKO TOMMILA

(Received for publication December 29, 1952)

The purification of inhibitors of virus hemagglutination has been the subject of many investigations. Purified inhibitors are especially needed for studies of the interaction between virus and inhibitors of virus hemagglutination. One rich source of virus hemagglutination inhibitors is eggwhite. It has been treated according to different methods which can be briefly if incompletely summarised as follows: Precipitation of the inhibitor at pH 5.7, extraction of the precipitate at pH 7.2 (4). The diluted eggwhite is centrifuged in an ultracentrifuge (4). Special filter paper is used to retain the inhibitor (1). Precipitation of the inhibitor with ovomucin at 0° C with distilled water and dispersion of precipitates in NaCl solution (2). Precipitation of the inhibitor with ethanol in cold (3, 4). Combination of the filter paper method with chloroform deproteinisation and precipitation with ethanol in cold (6). Electrophoretic separation of the active component from semi-purified inhibitor (5). Perhaps the best purification result was obtained by electrophoretic separation (purification factor 190), though the yield was small.

The writers have precipitated the inhibitor with distilled water in cold and for further purification used the ultracentrifuge. Inhibitor preparations are obtained easily and in satisfactory amounts by this method; they show purification factors (PF) from 100 to 400 and contain no measurable amounts of proteins.

[1] Aided by a grant from the Sigrid Jusélius Foundation.

Notes have been made of the properties of the inhibitor and some data are given on the precipitation of inhibitor with acetone in cold.

Methods. — The virus strain employed in the investigations was the Lee Strain of influenza B (F8 M139 E158—165). It was inactivated by heating the infected allantoic fluid at + 56° C for 30 minutes.

The inhibition activity of the preparations was titrated by first diluting the preparation 1/20 and making in buffered saline a twofold dilution series of it in 0.25 cc volume. 4 agglutinating units of inactivated virus were added to 0.25 cc and the mixture was allowed to remain at room temperature (about + 18° C) for 30 minutes after which 0.5 cc of 0.5 per cent chicken red cells was added. The results were read after an hour. The last dilution to inhibit completely the hemagglutination produced by the virus is adopted as the inhibition titre. Virus control was used to prove that the virus quantity in titration is 4 agglutinating units.

The nitrogen determinations of the preparations were made by the micro-Kjelldal method. The polysaccharide content was determined by the orcinol method. Viscosity determinations were made with Ostwald's viscosimeter at +25° C. Adsorption determinations were made with Beckman's spectrophotometer (Beckman Quartz Spectrophotometer, Serial No. 366) and a Spinco ultracentrifuge (Spinco ultracentrifuge Model L, Serial No. 151) was used for the ultracentrifugation.

The Preparation of Semi-purified Inhibitor. — Double its volume of +4° C distilled water was added at +4° C to 50 cc of eggwhite filtered through gauze stirring at the same time. The suspension was allowed to stand overnight at +4° C. The precipitate thus formed was separated by centrifugation at 3,000 r.p.m and washed three times with +4° C distilled water. Finally the precipitate was extracted with 0.06 M phosphate buffer of pH 7.2 for 24 hours at +4° C. After extraction the solution was centrifuged at 1,500 r.p.m for 15 minutes. By this means a viscous, slightly opalescent solution was obtained. (A part of the precipitate remained insoluble and could be re-extracted after centrifugation into a smaller quantity of buffer although the inhi-bition titre thus remained fairly low).

By way of example may be mentioned a semi-purified inhibitor thus prepared whose PF was relatively high. (Table 1).

TABLE 1

	Reciprocal of Inhibition Titre	N mg/ml	PF
Initial eggwhite 1: 10	1,280	1.930	
Semi-purified inhibitor	640	0.012	80

Simple precipitation with distilled water in cold gave an inhibitor with a PF as high as 80. The PF of the inhibitors prepared by the abovementioned method has varied slightly on each side of 50. To sum up, it is easy to produce inhibitors possessing a relatively high purification coefficient simple by using the method described.

Further Purification of Semi-purified Inhibitor by Ultracentrifuge. — Hardy and Horsfall (3) have shown that eggwhite inhibitor precipitates in the centrifuge. Lanni *et al.* (4) attempted purification by using as the initial substance eggwhite diluted 5 times in phosphate buffer and centrifuged once at 67,000 r.p.m. for 90 minutes and twice at 67,000 r.p.m. for 60 minutes. The sediment thus obtained was extracted in 0.06 M phosphate buffer (pH 7.2). By this method they arrived at 49 as the maximum PF attainable after the third centrifugation. Each centrifugation increased purification.

100 cc of semi-purified inhibitor (PF about 50) was centrifuged in an ultracentrifuge at 35,000 r.p.m. for 30 minutes (average centrifugal force = 80,730 $\times$ g). The number of revolutions mentioned and the centrifugation time have been found suitable for the sedimentation of an inhibitory active component; the nitrogenous impurity remains largely in the supernatant. The slight, transparent sediment which settled at the bottom of the centrifuge tubes was extracted to one-fifth of the original volume of 0.06 M phosphate buffer (pH 7.2) by stirring vigorously. Extraction was continued for 24 hours at + 4° C, stirring occasionally. A part of the sediment did not dissolve. It was centrifuged away at 1,500 r.p.m. for 15 minutes. Titration of the inhibition activity thus obtained was carried out after that and the nitrogen determination made by the above-mentioned methods. The nitrogen determination was not made of all the semi-purified inhibitors employed as the initial substance.

Table 2 gives four examples of inhibitors purified further with an ultracentrifuge. The first example also shows the values of the semi-purified inhibitor. The other examples only reveal the total purification on the basis of nitrogen.

TABLE 2

	Reciprocal of Inhibition Titre	Nitrogen mg/ml	PF
1. Eggwhite 1: 10	1,280	1.730	
Semi-purified	640	0.017	51
High purification inhibitor	640	0.007	124
2. Eggwhite 1: 10	1,280	1.781	
High purification inhibitor	1,280	0.014	127
3. Eggwhite 1: 10	640	1.781	
High purification inhibitor	640	0.005	362
4. Eggwhite 1: 10	1,280	1.729	
High purification inhibitor	640	0.002	432

Proteins cannot be demonstrated in the high purification preparation by means of sulfosalicylic acid. Also, it is no longer possible in spectrophotometric examination to observe the adsorption maximum characteristic of proteins at the 280 mμ, wavelength though it still emerges when semi-purified inhibitor is used. The preparation obtained may thus for practical purposes be considered free from nitrogenous impurities, in the first place proteins.

Koziński and Mikulaszek (6) obtained a preparation which contained 26% carbohydrates and 76% proteins with the purification method. The carbohydrate determination (orcinol method) made from a preparation purified by the writers in an ultracentrifuge showed the preparation to contain 0.5 mg/ml carbohydrates computed in glucose. According to dry weight determination the preparation contained no substances other than salts and carbohydrates.

Electrophoretic study of the purified inhibitor would have been of particular interest. However, despite attempts made, it remained without results for the reason that the preparation dissolves at too low a concentration.

One centrifugation only was used in the above-mentioned ultracentrifuge purification which gave a high degree of purification from nitrogenous impurities. It would obviously be possible in preparations in which the quantity of nitrogen remains bigger to reduce it by means of repeated centrifugations.

An interesting question is why the inhibition activity in native eggwhite is over 10 times that of purified preparations.

It may also be mentioned that the viscosity determinations made from an eggwhite dilution of 1: 10, from semi-purified and highly purified inhibitor, have confirmed the view held by Lanni *et al.*, (4) viz. that when the degree of purification on the basis of nitrogen advances the viscosity increases. This has been taken to mean that the inhibition activity follows the viscous component.

It can be said that it is possible to produce inhibitors of a high degree of purification and possessing a sufficient titre by means of the ultracentrifuge purification method used in the present work. The yield obtained with one centrifugation from an initial 100 ml of semi-purified inhibitor is 20 ml.

Attempts at Purification with Acetone Precipitation. — Hardy and Horsfall (3) by means of alcohol precipitation in cold obtained from eggwhite a fraction with a virus hemagglutination inhibitor activity on the basis of dry weight that was 5.4 times higher than that of the original eggwhite. Lanni *et al.* (4) obtained with a similar method of 60-fold purification from nitrogen and a 55-fold purification on the basis of dry weight.

Using acetone fractionation in cold the present authors tried to purify semi-purified inhibitor with a PF of 48 from nitrogen.

Five 10 ml lots of semi-purified inhibitor were precipitated with different volumes of acetone in cold. The acetone percentage was 18, 28, 37, 44 and 50. A white precipitate was formed in each case and it was allowed to stand overnight at +4° C. The precipitates were centrifuged apart the following day (3,500 r.p.m., 15 minutes). The precipitates were not easily soluble in 0.06 M phosphate buffer (pH 7.2). The supernatants were dialyzed for 48 hours against phosphate buffer to remove the acetone.

Table 3 gives the results obtained.

TABLE 3

THE ACETONE PRECIPITATION OF SEMI-PURIFIED INHIBITOR

(The semi-purified inhibitor: Reciprocal of inhibition titre 1280; N content 0,035 mg/ml; reciprocal of inhibition titre/N $\times$ 10^{-3} = 36; PF 48).

Percentage of Acetone	Supernatant				Precipitates
	Reciprocal of Inh. titre	N mg/ml	Recipr. of Inh.t./N. 10^{-3}	PF	Reciprocal of Inh. titre
18	640	0.015	43	56	80
28	640	0.008	80	105	80
37	320	0.005	64	84	80
44	160	0.003	53	70	80
50	80	—	—	—	80

As seen from the table, the different acetone quantities precipitated approximately evenly both the inhibitory component and nitrogenous impurities since both the inhibition titre and the nitrogen value decreased in proportion, perhaps with the exception of the 28% acetone concentration. The PF remained, of course, correspondingly approximately unchanged. Employing the largest quantity of acetone probably gave the maximum purification coefficient for the supernatant, but this is of no practical significance with such a low inhibition titre. Considerable changes (denaturation) may have occurred during precipitation of the inhibitory active components: different precipitates gave the same inhibition titre when extracted in the same volume (15 ml phosphate buffer) despite the fact that the inhibition titre of the supernatants continued to fall with increasing acetone quantity.

Hence, acetone precipitation did not prove a suitable purification method.

SUMMARY

Virus hemagglutination inhibitors possessing great purity on the basis of nitrogen were produced from semi-purified inhibitor in the present work by the ultracentrifuge method. The PF varied between 124—432. Acetone precipitation attempts with semi-purified inhibitor gave no practicable result.

REFERENCES

1. Csáky, T. Z., Lanni, F., and Beard, J. W.: Proc. Soc. Exper. Biol. and Med. 1950:*75:*105.
2. Gottschalk, A., and Lind, P. E.: Brit. J. Exper. Path. 1949:*30:*85.
3. Hardy, P. H., Horsfall, Fl. Jv.: J. Exper. Med. 1948:*88:*463.
4. Lanni, F., Sharp, D. G., Eckert, E. A., Dillon, E. S., Beard, D., and Beard, J. W.: J. Biol. Chem. 1949:*179:*1275.
5. Sharp, D. G., Lanni, F., Lanni, Y. T., Csáky, F. Z., and Beard, J. W.: Arch. of Biochem. 1951:*30:*251.
6. Koziński, A. W., and Mikulaszek, A.: Med. Boswiadczane i Mikrobiol. 1951:*3:*42. Cited from Chem. Abstr. 1952:*46:*5654.

FROM THE DEPARTMENT OF VIRUS RESEARCH, UNIVERSITY OF HELSINKI

THE OCCURRENCE OF THE COXSACKIE GROUP OF VIRUSES IN FINLAND[1]

by

N. OKER-BLOM and PIRKKO POHJANPELTO[2]

(Received for publication December 31, 1952)

Since Dalldorf's original report (4) in 1947, Coxsackie viruses have been isolated in many parts of the world, including Denmark and Sweden, and many interesting data concerning this new group of viruses have accumulated during the last few years (1, 3, 5, 6, 9, 10).

This report is therefore mainly intended to add to the knowledge of the geographical distribution of the Coxsackie viruses on the basis of virus isolations from some patients with paralytic poliomyelitis and aseptic meningitis during an epidemic of poliomyelitis in Finland in the autumn 1950. Furthermore some studies on the antibody level in patients and normal controls, using the local strain of virus in complement fixation tests, have been undertaken.

METHODS

All the specimens were stored at — 20 C until used.

Virus Isolation. — A 25 per cent suspension of faeces was made in destilled water or physiological saline solution and run in an angle centrifuge for 20 minutes at 18.000 rpm. One thousand unites of penicillin and 5 mg of streptomycin were added for each ml of the supernatant fluid. 0.02 ml of this mixture was inoculated intraperitoneally into two litters of suckling mice 1 to five days of age.

[1] This Investigation was aided by a grant from Sigrid Jusélius Stiftelse.
[2] With the technical assistance of Miss A. Jokinen.

Immune Sera. — These were produced in adult mice by inoculating intraperitoneallyy twice a week 0.2 ml of virus suspension. The first time the virus suspension was diluted 1 in 100, the second time 1 in 50 and the two last times 1 in 10. The mice were bled either by cardiac puncture or by cutting the fore limb seven days after the last immunisation.

Antigen. — The antigen used was protamine treated antigen prepared according to the method given by Kraft and Melnick (8).

Complement Fixation Technique. — A modified Kolmer test was used (7). The complement used was pooled guinea pig serum. It was standardized so that 0.2 ml contained 1.26 full unites. The hemolytic system consisted of equal parts of 2.5 per cent sheep red cells and the appropiate dilution of the hemolysin containing two full units in 0.2 ml. Sensitisation proceeded at room temperature for 30 minutes. Serial twofold dilutions of serum were made and 0.2 ml of each dilution was placed in a single tube followed by 0.2 ml antigen and 0.2 ml complement. The tubes were then incubated at 4°C for 15 to 20 hours. At the end of that time 0.4 ml of sensitized red cells were added and hemolysis was allowed to take place in a water bath at 37°C until the antigen control was clear in about 20 to 30 minutes. Readings were made after the tubes had been standing over night at 4°C temperature. The colour of the fluid over the sedimentated red cells was then compared to colour standards made of hemolyzed red cells. The endpoint of a titration was 50 per cent hemolysis.

Neutralisation Test. — A constant dose of 200 ID_{50} of the virus was mixed with equal parts of the test serum diluted 1: 5, 1: 10 and 1: 50, thus giving final serum dilutions of 1: 10, 1: 20 and 1:100 and a final virus dose of 100 ID_{50}. These mixtures and a virus control containing 100 ID_{50} of virus were allowed to stand one hour at room temperature and inoculated intraperitoneally into one litter of suckling mice. If half or more of the mice inoculated with a certain serum dilution remained healthy this dilution was counted as the neutralisation titer of the serum.

Virus Typing. — Neutralisation tests were performed with unknown virus and known Coxsackie immune sera and correspondingly with known Coxsackie virus strains and unknown immune serum.[1]

[1] We wish to express our grateful appreciation to Professor Sven Gard of the Department of Virus Research, Karolinska Institutet, Stockholm, Sweden, for the Coxsackie type strains and immune sera.

RESULTS

Virus Isolations. — Virus was isolated from the stools of one out of five patients with paralytic poliomyelitis and from two out of five patients with aseptic meningitis. The strains belong to Dalldorfs type 10 group A.

Antibody Titrations. — Because of the shortage of mice the antibody titrations have been performed using the complement fixation technique in the following manner. For each titration a homologous mouse immune serum served as a control. All the patients sera showing some inhibition were titrated once more with the virus antigen and as a further control with antigen prepared in the same manner from normal suckling mice of the same age. Only the sera which showed inhibition at least in a serum dilution of 1: 4 in both titrations with the virus antigen but no inhibition with the normal antigen were counted as positive.

Although the complement fixation test has been used previously for similar studies (2, 8) it was desirable to compare the complement fixation test and the neutralisation test. For this reason 7 patients sera and 4 normal sera were titrated both with complement fixation and with the neutralisation method (table 1). The results obtained by these methods seem to correspond roughly each other. Thus the results obtained with complement fixation tests seem to be fairly reliable.

The results of titrations of patient sera, including 40 patients with paralytic poliomyelitis, 14 patients with aseptic meningitis and 18 patients with various acute infections with neurological symptoms, and of 92 sera from healthy individuals are shown in table 2. The ages of the patients ranged from a few months to 40 years. Ca. 60 per cent of the patients were below 15 years of age. The 92 sera from healthy individuals consisted of 72 adult sera from medical students and from laboratory personnel 20 to 40 years of age not working in the department where Coxsackie virus studies were carried out, and of 20 sera from children 2 to 11 months of age. The normal sera were collected during the year following the epidemic of poliomyelitis. There was no difference between the hight og the titer in the patient sera and in the normal sera respectively. The titers were as a rule low, mostly 1/4 and 1/8. From the

TABLE 1

COMPARISON BETWEEN THE COMPLEMENT FIXATION TITER AND THE NEUTRALISATION TITER OF SOME PATIENT SERA AND NORMAL SERA

	Pathological Sera							Normal Sera			
	V.E.	E.K.	M.Y.	H.T.	K.L.	R.P.	P.P.	1	2	3	4
C. F. titer[1] ..	64	32	16	8	—	—	—	8	—	—	—
Neutr. » [1] ..	>100	>100	20	10	—	—	—	>100	—	—	—

TABLE 2

DISTRIBUTION OF POSITIVE COMPLEMENT FIXATION TESTS AMONG PATIENTS WITH PARALYTIC POLIOMYELITIS, ASEPTIC MENINGITIS AND VARIOUS ACUTE INFECTIONS, AND HEALTHY ADULTS AND CHILDREN

Sera	No. of Tests	No. of Positive Tests	Positive Tests Per Cent
Pathological sera			
Paralytic poliomyelitis	40	14	35
Aseptic meningitis.....	14	5	35
Various acute infections with neurological symptoms ..	18	5	28
Normal sera			
Adults	72	18	25
Children	20	0	0

three patients, who were proved to harbour Coxsackie virus two showed antibody response against their own virus. From the third patient no proper serum specimen was obtained.

SUMMARY AND CONCLUSION

During an epidemic of poliomyelitis in Finland in the autumn of 1950, Coxsackie viruses belonging to Dalldorf's type 10 group A have been isolated from the stools of one out of five patients with paralytic poliomyelitis and from two out of five patients with aseptic meningitis.

Antibody studies using the local virus strain in complement fixation tests have shown the presence of complement fixing antibodies in 35 per cent of the sera of patients with paralytic polio-

[1] The titers are expressed as the reciprocal of the serum dilutions.

myelitis, in 35 per cent of the sera of patients with aseptic meningitis and in 28 per cent of the sera of patients with various acute infections with neurological symptoms during the same epidemic.

Complement fixing antibodies could be demonstrated in 25 per cent of the sera of healthy adults collected the year following the epidemic. In sera from healthy children 2 to 11 months of age collected at the same time no complement fixing antibodies could be demonstrated.

In some cases the complement fixation tests were controlled by neutralisation tests. The results obtained by the two methods corresponded roughly each other. The complement fixation test, therefore, seems to give a fairly reliable notion of the antibody level in Coxsackie virus infections.

Thus, on the basis of these studies, infections with the Coxsackie group of viruses seem to be fairly widespread in Finland, too, and, of the diseases here studied, apparently most often related to paralytic poliomyelitis and aseptic meningitis.

REFERENCES

1. Beeman, E. A., Huebner, R. J., and Cole, R. M.: Am. J. Hyg., 55:*83:*1952.
2. Casals, J., and Olitsky, P. K.: Fed. Proc. 9:*570:*1950.
3. Curnen, E., Shaw, E. W., and Melnick, J. L.: J.Am. Med. Assn., 141:*394:*1949.
4. Dalldorf, G., and Sickles, G. H.: Science, 108:*61:*1948.
5. Dalldorf, G.: Fed. Proc., 9:*570:*1950.
6. Gabinus, O., Gard, S., Johansson, T., and Pöldre, A.: Svenska läkartidn. 49:*2225:*1952.
7. Kolmer, J. A., and Boerner, F.: Approved Laboratory Technic. Fourth edition. D. Appleton Century Company, Inc., New York and London 1945.
8. Kraft, L. M., and Melnick, J. L.: J. Exper. Med. 92:*483:*1950.
9. Symposium on the Coxsackie-group of Viruses. Second International Poliomyelitis Conference. Copenhagen 1951.
10. Vivell, O., and Gädeke, R.: Erg. Hyg. Bakt. Immunol. u. exper. Ther. 27:*512:*1952.

FROM THE DEPARTMENT OF FORENSIC MEDICINE, UNIVERSITY OF HELSINKI

INFLUENCE OF CORTISONE ON MITOSIS[1]

I

EFFECT OF SINGLE DOSE AND PROLONGED APPLICATION

by

H. TEIR and A. ISOTALO

(Received for publication January 10, 1953)

According to a theory from our growth laboratory, the formation of new cells in the tissues is probably, at least in higher vertebrates, controlled by two humoral systems (13, 14, 15). According to this theory a peripheral system is represented, inter alia by the mitosis-stimulating factor which is activated during tissue degeneration both *in vivo* (13) and *in vitro* (14, 15). The other system is of a superior nature and here the growth hormone of the anterior lobe of the hypophysis, the adrenal gland, the thyroid gland, the gonads and possibly even other known internal secretory organs may be of importance.

When investigating the importance of a superior humoral system in the formation of new cells it is reasonable to take as a starting point the adrenal glands, since the part these play has been investigated by several authors (1, 2, 3, 4, 5, 6, 7, 9, 10, 11) during recent years. Most investigators have established that administration of cortisone lowers the mitotic ratio. Since the skin is among the labile elements in which there is a continuous physiological wearing out of cells and consequently also a continuous formation of new cells, it is a useful object for the study of mitosis in general. We therefore chose the skin as one object of investigation.

[1] An investigation aided by a grant from the Sigrid Jusélius Foundation, Helsinki, and an institutional grant from the Damon Runyon Memorial Fund, New York.

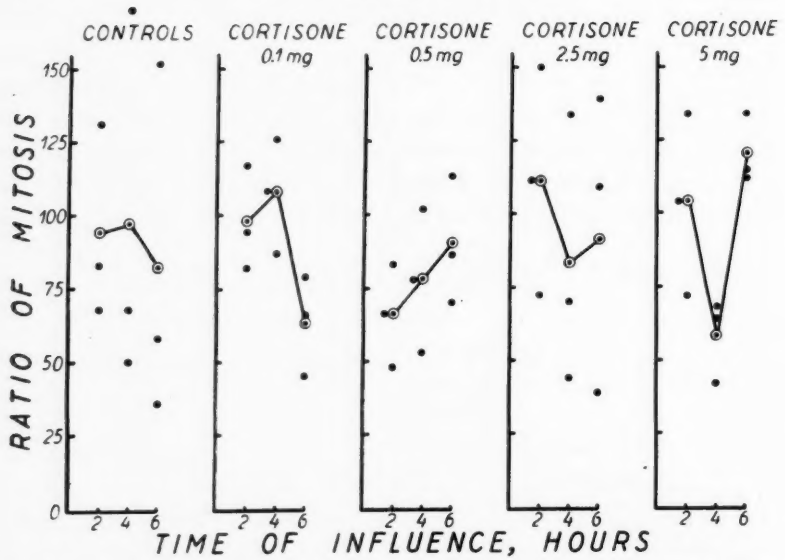

Fig. 1. — *Orbital gland.* Mitotic ratio of 3-week-old rats after single small doses of cortisone. The rings indicate the mean values of each group.

Another reason for our choice was that the effect of cortisone on the skin has already been studied with the skin of the rat (11) and the mouse (1, 2, 3, 6, 7) as objects of investigation. In order to investigate also a stabile organ in which mitotic cell division takes place only during the growth period of the animal, ending when the growth period ends, we chose the orbital gland for our second object of investigation.

METHODS

As experimental animals, white rats of the Wistar strain were used. In order to find mitoses in the outer orbital gland the effect of single doses were tested on 1-month-old rats chosen so that their body weights were about the same. For the same reason prolonged cortisone treatment was started as early as at the age of 18 days. A total of 153 rats were used. They all received the same treatment. Four days prior to treatment and onwards they received a food mixture consisting of 69 per cent sucrose, 20 per cent casein, 5 per cent rape oil, 4 per cent of a salt mixture and 2 per cent of a vitamin mixture. The salt mixture consisted of NaCl 10.6 per cent, $MgSO_4 7H_2O$ 11.2 per cent, Na_4HPO_4 6.4 per cent, K_2HPO_4 31.8

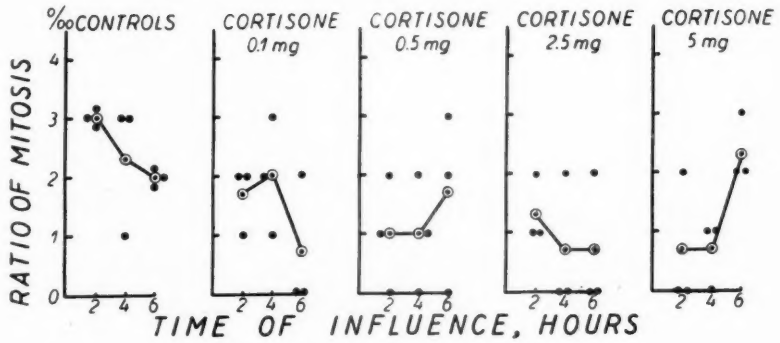

Fig. 2. — *Skin.* Mitotic ratio in epidermis of 3-week-old rats after single small doses of cortisone.

per cent $CaHPO_4 7H_2O$ 32.0 per cent, Ca-lactate $5H_2O$ 7.5 per cent, Ferri citr. 0.43 per cent and KJ 0.07 per cent. The vitamin mixture consisted of yeast 87.5 per cent and cod liver oil 12.5 per cent. The rats had free access to food, which was given twice a day. The temperature in the animal house was kept fairly constant between 18 and 20 C°.

The cortisone preparation used was Merk's »Cortene». To obtain small doses it was diluted with physiological saline solution. As single doses 50, 25, 10, 5, 2.5, 0.5 and 0.1 mg of cortisone were used and injected intraperitoneally. For prolonged administration 2.5, 0.5 and 0.1 mg of cortisone were given intraperitoneally once a day.

The rats were decapitated at the same time of day, and the skin and the outer orbital gland were immediately fixed in Bouin's solution.

The mitotic ratio in the skin was established by counting the number of mitoses per 2000 cells and expressing the result as mitoses per thousand cells. In the orbital gland, however, the mitotic ratio was determined in the same way as before (14). With binocular × 10 and objective 40 the number of mitoses in 100 fields of vision was determined. The mitotic value thus obtained was used as an index of the mitotic ratio.

INFLUENCE OF A SINGLE DOSE OF CORTISONE

Small Doses. — A group of 45 3-week-old rats were divided into 5 groups of 9 animals each. One group served as a control and in

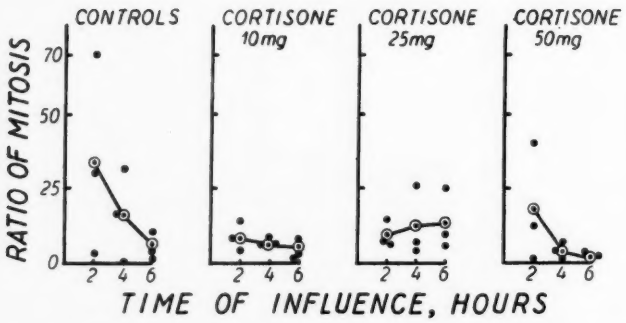

Fig. 3. — *Orbital gland.* Mitotic ratio of 1-month-old rats after single large doses of cortisone.

this group the animals were given 0.5 ml of saline solution intraperitoneally. In the same way the animals of the other groups received cortisone in doses of 0.1, 0.5, 2.5 and 5 mg mixed with 0.5 ml of saline solution.

The animals were killed in groups of 3 animals from each experimental group 2, 4 and 6 hours after treatment.

In the case of the orbital gland the cortisone hardly had any effect upon the mitotic ratio (Fig. 1). A decrease of the mitotic ratio was obtained at 4 hours with 2.5 and 5 mg only. In the skin, however, the ratio of mitosis was very low and appeared to be inversely proportional to the dose of cortisone used (fig. 2).

Large Doses. — Since the effect of the cortisone on the mitotic ratio in the orbital gland was so insignificant, doses of 10, 25 and 50 mg of cortisone were given in the next experiment on 34 4-week-old rats. The experimental animals were also now decapitated 2, 4 and 6 hours after injection. One group received saline solution only.

The animals which received 50 mg cortisone underwent, within the first five minutes, a deep shock that lasted for about 45 minutes. After about two hours there was a new stage of exhaustion that persisted throughout the experiment. The animale moved only with difficulty, and seemed to be very ill. Those that received 25 mg of cortisone also showed symptoms of shock during the first hour after injection.

The mitotic ratio in the orbital gland was fairly low in the control group, decreasing further during the course of the experiment

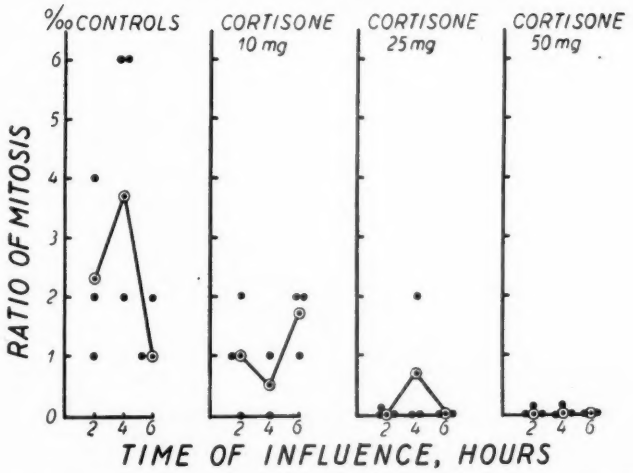

Fig. 4. — *Skin.* Mitotic ratio in the epidermis of 1-month-old rats after single larger doses of cortisone.

(Fig. 3). In the cortisone-treated rats mitoses were also found even though their number was lower than that in the controls at the beginning of the experiment. Mitoses were even found in the 50 mg group of shock-influenced rats.

There was an obvious decrease in the mitotic ratio in the skin after cortisone treatment (Fig. 4). In the 25 and 50 mg groups mitoses were found only in some individuals. The mitotic ratio for most of them was 0. In this experiment also, the mitotic ratio was inversely proportional to the dose of cortisone administered.

Since this experiment did not give a perfectly clear picture of the effect of large doses of cortisone on the mitotic ratio in the outer orbital gland, another similar experiment comprising 30 4-week-old rats was performed. These were divided into 3 groups. One group received saline solution, and the two others 10 and 25 mg of cortisone. The animals were decapitated 2, 4 and 6 hours after injection.

The mitotic ratio in the outer orbital gland was here not influenced by cortisone (Fig. 5); it was even somewhat higher in the treated animals than in the controls. In the skin, however, an obvious decrease of the mitotic ratio was obtained, the mitotic value being 0 in several animals (Fig. 6). The peak 4 hours after the application of 10 mg of cortisone cannot be interpreted as an individual peculiarity since the mitotic ratio was fairly high in all three animals, viz. 5, 4 and 2.

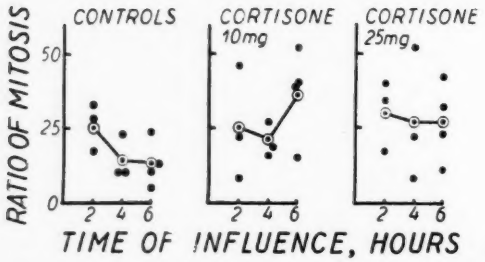

Fig. 5. — *Orbital gland.* Mitotic ratio of 1-month-old rats after single larger doses of cortisone.

INFLUENCE OF REPEATED DAILY CORTISONE INJECTIONS

In an experiment comprising 44 rats cortisone was administered intraperitoneally once every morning from the age of 18 days onwards. The animals were divided into 4 groups; one was given saline solution and the others 0.1, 0.5 and 2.5 mg of cortisone. The animals were killed exactly one day after the last injections 5, 10, 15 and 20 days after the commencement of treatment. The body weights in gr were as follows:

	5 days	10 days	15 days	20 days
Saline solution:	25, 27, 27	35, 38, 35	39, 50, 43	40, 72, 52
Cortisone 0.1 mg:	15, 16, 15	28, 32, 30	46, 50, 48	33, 48, 40
» 0.5 mg:	27, 33, 30	40, 46, 43	29, 32, 29	30, 65, 54
» 2.5 mg:	20, 28, 20	22, 31, 27	40, 42.	

The mitotic ratio in the outer orbital glands appears from Fig. 7. There is no distinct difference with regard to the control animals. One rather gets the impression that 10 days from the beginning of treatment and onwards the mitotic ratio was higher in the cortisone-treated rats than in the controls.

A similar results was obtained in the skin (Fig. 8). Here also the highest mitotic values were observed in the group to which the highest cortisone dose, 2.5 mg, was administered.

DISCUSSION

With cortisone a markedly antimitotic effect was obtained in the skin of the rat just as was reported by Studer and Frey (11). The ratio of mitosis was often inversely proportional to the dose of cortisone administered. The effect was at its highest when the dose was large enough to bring about an obvious shock-condition and this observation is thus in conformity with previous experiences

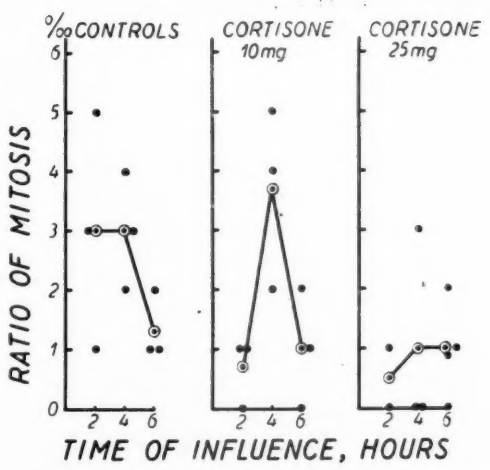

Fig. 6. — *Skin.* Mitotic ratio in the epidermia after single larger doses of cortisone.

of the influence of shock (3, 6) and stress (1, 2) on the mitotic ratio in the skin of the mouse. A similar effect on the mitotic ratio in the latter tissue was also obtained with ACTH (5).

The negative, or very insignificant effect of cortisone on the mitotic ratio in the outer orbital gland of the rat was somewhat surprising. Even in animals which received as large doses as 50 and 25 mg of cortisone, this substance often had no effect whatsoever on the mitotic ratio. This result corresponds to the negative effect that cortisone proved to have on the mitoses in the small intestine and in the proliferation obtained with thio-uracil in the thyroid gland (10).

Whether the effect of cortisone on the mitoses in the skin is direct, or whether it takes its effect through some other internal secretory system did not appear from our investigations. It has been proved that epidermal mitotic activity is dependent on active energy production through the tricarboxylic acid cycle (4). Shock results in an absolute decrease in the mitotic ratio in the skin (3, 6). In this reaction adrenaline and the glucocorticoid hormone play a part (7, 9). Disturbances in the carbohydrate metabolism are an important factor in the shock condition [see Bullough (1)]. The mitotic ratio in the skin is thus influenced by the general carbohydrate metabolism of the body. Since, however, the mitotic ratio in the orbital gland and the intestinal epithelium are not influenced

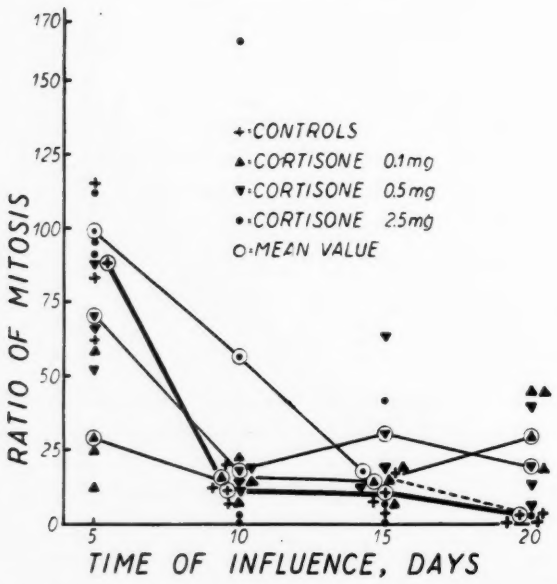

Fig. 7. — *Orbital gland.* Mitotic ratio after daily administration of 18 days.

by cortisone and shock, it might be assumed that mitotic cell division in different tissues has a different chemical background. This is, in fact, rather natural, since it would be remarkable if the cells, while preparing for mitosis and during actual cell division were to lose their specific chemical properties.

There is also a difference in the reaction of different tissues with regard to colchicine. Even in the same tissue, cells belonging to different nuclear classes exhibit different latent periods with regard to the colchicine effect (1 d, 14).

We did not observe any sex-conditioned difference in the cortisone effect. The same result was obtained with regard to the skin of the rat (11).

The daily administration of 0.1, 0.5 and 2.5 mg of cortisone from the age of 18 days and onwards did not disturb the rhythm of mitotic cell division in the skin or in the orbital gland, since one day after the last of 5, 10, 15 and 20 injections the mitotic ratio in these organs was almost normal. The antimitotic effect of cortisone can therefore hardly be anything but incidental and of a very temporary nature.

By long-continued stress induced by overcrowding, Bullough (1)

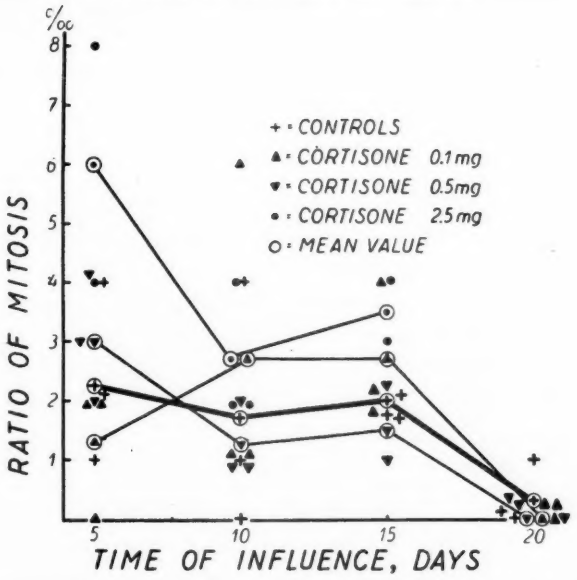

Fig. 8. — *Skin.* Mitotic ratio after daily administration of cortisone from the age of 18 days.

caused considerable increase in adrenal size comprising both the cortex and the medulla. He assumed that the mitotic depression observed was caused by an excessive secretion of adrenalin from the medulla and by the glucocorticoid hormone from the cortex. Both these hormones have an antimitotic effect. In our experiments the animals receiving daily doses of 2.5 and 0.5 mg of cortisone showed symptoms of stress during the first few days of the treatment but later on cortisone did not seem to affect them, a fact proved, for instance, by a steady gain in weight. The same kind of adaptation could be noticed with regard to the mitotic ratio.

The investigation thus argues in favour of our growth theory (13) of the existence of a superior humoral system of control of somatic cell division. The function of the adrenal gland certainly occupies a fairly central position in this system. It seems, however, to influence the mitotic cell division of different tissues in different ways. An antimitotic effect in the skin has been obtained not only by adrenalin and by the glucocorticoid hormone, but also by adrenochrome (1). The mineral corticoid hormone, deoxycorticosterone acetate, however, has no effect on mitoses in the skin of the mouse (1). In this connection the possibility of inducing cortical cell

division with ACTH only (1), and the assumption that the secretion of ACTH is followed by a secretion of the hypophyseal growth hormone (8) are interesting.

SUMMARY

1. A clear antimitotic effect was obtained in the skin of the rat with cortisone. The ratio of mitosis was often inversely proportional to the dose of cortisone used.

2. The cortisone was found hardly to influence the mitotic ratio in the outer orbital gland of the rat at all.

3. The varying effect of cortisone on the mitoses in different tissues was assumed to depend on differences in the chemical background of the mitoses in different tissues.

4. Prolonged administration of cortisone did not disturb the mitotic rhythm in the skin and in the outer orbital gland.

5. These experiments, as well as previous investigations on the influence of adrenal hormones on somatic cell division, argue in favour of the correctness of our growth theory. According to this theory the mitotic cell division and the multiplication of cells in general in the tissues are controlled, on the one hand, by a superior humoral system in which several internal secretory organs cooperate, and, on the other, by a peripheral control system in the tissues.

REFERENCES

1. Bullough, W. S.: J. Endocrinol. 1952:*8:*265.
2. Bullough, W. S.: J. Endocrinol. 1952:*8:*365.
3. Bullough, W. S., and Green, H. N.: Nature 1949:*164:*795.
4. Bullough, W. S., and Johnson, M.: Proc. Roy. Soc. B. 1951:*138:*562.
5. Green, H. W.: Brit. Med. J. 1950:*1:*1165.
6. Green, H. W., and Bullough, W. S.: Brit. J. Exp. Path. 1950:*31:*175.
7. Green, H. N., and Ghadially, F. N.: Brit. Med. J. 1951:*1:*696.
8. Li, C. H.: In Symposium on Pituitary-Adrenal Function. Washington: Amer. Ass. Adv. Sci. 1951
9. Stoner, H. B., and Green, H. W.: Brit. J. Exp. Path. 1950:*31:*603.
10. Studer, A.: Zeitschr. f. Rheumaforsch. 1950:*9:*237.
11. Studer, A. R., and Frey, J. R.: Dermatolgica 1952:*104:*1.
12. Teir, H.: Acta Pathol. et Microbiol. Scand. *Suppl. 54*, 1944.
13. Teir, H.: Comm. Biol. Soc. Scient. Fenn. 1951:*13:*1.
14. Teir, H.: Acta Pathol. et Microbiol. Scand. 1952:*30:*158.
15. Teir, H.: Growth 1952:*16:*85.

FROM THE DEPARTMENT OF FORENSIC MEDICINE, UNIVERSITY OF HELSINKI

INFLUENCE OF SOME MINERALS ON THE GROWTH AND TRANSPLANTABILITY OF THE ITB-TUMOR OF THE WHITE RAT[1]

by

H. TEIR, K. PYÖRÄLÄ, and A. R. ALHA

(Received for publication January 12, 1953)

Some three years ago a rumour spread in this country to the effect that positive results had been obtained in the treatment of malignant tumors with an extract made from the ash of birches growing in the Kalajoki valley. The information attracted great attention even to the extent that this method of treatment came to be used beyond the frontiers of Finland. Since the treatment has mainly been given by laymen, the true nature of the cases treated and the results of treatment have perhaps not always been judged with sufficient objectivity. This question should therefore be investigated with the usual scientific critique and first, perhaps, by animal experiments before experiments are made on sick persons. The object of this investigation has therefore been to investigate the effect of birch ash on experimental transplantable cancer of the rat.

The results of investigations on the mineral metabolism of neoplasms are very contradictory. It has been maintained, however, that a certain deviation from normal conditions has been established in the mineral metabolism within the sphere of general fluid balance

[1] An investigation aided by a grant from Orion Medical Factory, and the Sigrid Jusélius Foundation, Helsinki and an institutional grant from the Damon Runyon Memorial Fund, New York.

(1, 2, 4). In the few investigations, however, which deal with the occurrence in neoplasms of rare minerals and their use in the treatment of tumors, nothing noteworthy has been established with certainty (1). Birch ash is known to contain a great variety of rarer mineral compounds besides some of the commoner metal salts, according to the soil.

To obtain a better idea of the effect of such substances on the growth and transplantability of the neoplasm, we used alongside with birch ash a factory-made mineral mixture which, as compared with birch ash, has the advantage of being constant to its composition.

MATERIAL AND METHODS

The birch ash was obtained from the Kalajoki valley. By semiquantitative spectroscopic analysis its percentage composition was determined as follows: $\geq$ 1: Al, Ba, Ca, K, Mg, Mn, Na, P. — 0.1: Zn. — 0.01: Co, Fe. — 0.001: Ag, Cu, Sn, Ti, T. — $<$ 0.001: Cr, Mo, Ni. — 0.001 — 0.0001: Pb. — ?: Rb, Sb, Sr. — There was no As, Be, Bi, Cd, Ga, Ge, Hg, In, Tl, Y, Zr.

The mineral mixture used for these experiment was the mass used for the production of Miral tablets (Orion), whose constituents were as follows: 100 mg of Miral mass contains: 6.4 mg of ferrotartrate, 1.6 mg of magnesium glycerophosphate, 1.6 mg of manganoglycerophosphate, 0.6 mg of cuprisulfate, 1.2 mg of zinc sulfate, 0.13 mg of cobalt chloride, 0.12 mg of nickel sulfate, 0.01 mg of potassium iodide, 0.1 mg of ammonium molybdate, 0.01 mg of sodium fluoride. These substances are mixed with a binding medium containing rice starch, talc and magnesium stearate.

In all experiments female rats of the Wistar strain, aged 4 to 7 months, were used. During the experiments each rat received 50 g of a dry food mixture daily. The percentage composition of the food mixture was as follows: Wheat bran, oatmeal, rye-flour and oat bran 22.2 each, casein 4.3, butter 2.2, yeast 2.2, calcium lactate 1.0, salt 0.7 and cod-liver oil 0.8.

It was considered desirable to use for the experiment a rapidly growing neoplasm in connection with which there would occur in some cavity of the body an exudate containing tumor cells capable of multiplication so that the direct effect of the minerals on them

could be observed. Such a tumor is the ITB- tumor developed in our laboratory as follows: The skin of the interscapular region is continuously painted twice a week with a 0.3 per cent solution of methylcholanthrene benzene and simultaneously an extract made of the skin of the rat is injected intraperitoneally (5). There will then develop in the peritoneal cavity of the rat a very infiltrating malignant tumor which obviously originates in the endothelium of the cavity. The tumor can easily be transplanted by means of a suspension of tumor tissue and when this is done intraperitoneally there develops in the peritoneal cavity of the rat a tumor proving lethal in 3 to 4 weeks. The tumor infiltrates the intestinal mesentery diffusedly and accumulates particularly abundantly in the region of the porta hepatis. More rarely large solid tumors occur. In connection with the tumor there is 10 to 100 ml of ascites which is sometimes hemorrhagic, particularly in connection with solid tumor nodes. Histologically the tumor tissue is formed of rather big cells which are pressed close together and, in places, arranged in an epithelium-like manner. There is very little stroma. Mitotic cell division is extremely active. Here and there, as is generally the case in rapidly growing tumors, there are necrotic areas, inflammation and hemorrhage. In the ascites up to 40 per cent of the cells are tumor cells, which often divide mitotically. The rest of the ascites cells are mainly lymphocytes and mesothelial cells.

For this investigation transplantation of the tumor was performed in the following way: About 20 g of tumor tissue from one rat was mashed, mixed with 30 ml of ascitic fluid from the same rat and diluted with 50 ml of saline solution. Each rat received 1 ml of this suspension. The tumor strain used originated from the rat ITB 162 (6).

Experimental Series No. 1. — In this series the effect of birch ash and of the mineral mixture given with the food was investigated. In the treatment of malignant tumors in man the daily dosage recommended has been 9 g of birch ash dissolved in 150 ml of water by boiling. The residue should be filtered away. It was, however, considered better to use birch ash as such in the experiments in order to include the slowly dissolving substances. The dose chosen was 1/50 of the human dose, which is equivalent to 180 mg of birch ash mixed daily with the food of each rat. According to the directions of the medical factory a daily dose of 270 mg of the mineral

mixture is recommended for man. A daily dose of 5 mg of this mixture was given to the rat with the food, this also being about 1/50 of the human dose.

For the experiments a total of 60 rats were used, divided into three groups of 20 each. The first group acted as controls and received ordinary food only, the second group received birch ash with the food and the third mineral mixture. After 9 days, transplantation of the ITB-tumor was performed from a rat belonging to the first transplantation generation of the strain used. 20 days after transplantation all animals were killed, macroscopic observations were made and specimens for histological examination were taken. Smear preparations were made of the ascitic fluid of 6 control animals, 5 animals from the ash group and 4 from the mineral mixture group. The preparations were stained according to Papanicolaou's method and cell differentiation was performed by counting a total of 400 cells.

Experimental Series No. 2. — In this series birch ash and the mineral mixture were administered intraperitoneally. The intention was to elucidate the direct effect of the birch ash and the mineral mixture on the tumor tissue by injecting it into the peritoneal cavity of the rats in which an ITB-tumor was already growing. In this way a higher concentration near the tumor tissue of the minerals, some slow in dissolving and slowly absorbed by the intestine, would be obtained.

For the injections homogeneous suspensions were used. The ash suspension was prepared as follows: Into 100 ml of distilled water 10 g of birch ash were stirred, a considerable portion of it remaining undissolved. To this suspension 10 ml. of 2—N HCl was added, the *pH* then being 7. When the suspension had stood 2 days the *pH* was 8. No second neutralization was performed, however. Of the suspension 0.7 ml (60 mg of ash) were injected into each rat daily. The mineral mixture suspension was prepared by mixing 200 mg of Miral mass with 100 ml of distilled water, a small portion of it remaining undissolved. Of this suspension 1 ml (2 mg of mineral mixture) was injected into each rat daily.

For this experiment a total of 25 rats were used. 5 normal rats received an injection of ash suspension and another 5 rats a suspension of the mineral mixture. These groups acted as controls and principally served to show any possible irritating and destructive

effect of the suspensions on the tissues. The animals were killed 11 days after the injection and macroscopic and histological examination were made. The remaining 15 animals were rats into which ITB-tumor from the rat ITB 162 had been successfully transplanted 18 days prior to the experiment. These rats were now divided into groups of 5 rats each. The first group was the actual control group and received distilled water intraperitoneally, the second groupl received ash suspension and the third a suspension of mineral mixture. 6 days later 5 ml of ascites were taken by puncture from each of the 15 animals and the cells were counted as in the experimental series no. 1. After 10 days 1 animal died of those that had received ash suspension. All animals were then killed, each of them having received 9 injections, and macroscopic and histological examinations were made.

RESULTS

Experimental Series No. 1. — Both in the control series and the mineral mixture series 16 rats (80 per cent) and in the birch ash series 17 rats (85 per cent) developed a tumor. No clear difference could be observed between the different groups in the development of the tumor, nor in the macroscopic or microscopic picture. In all cases in which the transplantation had been successful, there was a typical ITB-tumor. Neither could any clear difference be noticed in the number of tumor cells in the ascitic fluid from the different groups, or in the mitotic ratios.

Experimental Series No. 2. Normal Rats. — In the birch ash group there was much unresorbed residue of ash. The parietal and the visceral peritoneum were reddish and swollen, in places covered by fibrinous accumulations. Between the intestinal coils there were frequently adhesions. Microscopically, acute inflammation could be observed, the connective tissue component dominated and of the inflammatory cells lymphocytes were more common than granulocytes. In places the peritoneum was thickened even to 15 times its normal thickness. In the mineral mixture group nothing abnormal could be established macroscpically. Microscopically a very slight inflammation in the visceral and the parietal peritoneum was observed.

Tumor Rats. — No other macroscopic differences were noticed

between the control, the ash and the mineral mixture groups than that the tumor tissue seemed more necrotic in rats of the ash group than in those from other groups. In the ash and the mineral mixture groups the same inflammatory changes were observed as in the normal rats. Histologically the tumors in all groups were typical ITB-tumors between which no noticeable differences could be observed. In all of them fairly active cell division could be observed. As regards the number of tumor cells and the mitotic ratio of the ascitic fluid, no differences between the different groups could be discerned. True, in the inflammatory cells of the ascites the relative proportion of lymphocytes and granulocytes had increased a little in the ash and the mineral mixture groups, the mesothelial cells being less conspicuous.

DISCUSSION

The investigation has shown that the birch ash from Kalajoki does not prevent the growth of the malignant ITB-tumor in the abdominal cavity of the rat. This appeared both from experiments in which ash was administered together with the food both before transplantation of the tumor and during its growth, and from those in which a suspension of birch ash was injected intraperitoneally into rats in which a tumor was already forming. The results were the same in parallell experiments with a factory made mineral mixture.

Active mitotic division and multiplication of the malignant cells both in the tumor and in the ascitic fluid argues in favour of high resistence of the tumor. It is interesting to compare the negative result of this investigation with Bullough's statement (3) that the mineral corticoid hormone of the adrenal gland, desoxycorticosterone acetate, does not have an antimitotic effect on the skin of the rat, as has cortisone.

In the experimental series in which a suspension of ash was injected into the abdominal cavity, necroses were found in the tumor tissue. In a control series in which a similar suspension was injected into the normal abdominal cavity, considerable inflammation and thickening of the peritoneum was established. It has earlier been found that minerals stimulate the autolytic processes of the tumor (1). It has not been possible, however, to destroy

malignant growth in this way. In our opinion birch ash might perhaps sometimes, by its necrotizing effect, have a favourable influence on tumors which cause obstruction of the intestinal passage.

Since in this investigation the effect of birch ash on malignant tumors in experimental animals was investigated, the negative results obtained do not allow definite conclusions to be drawn with regard to the effect of this agent on human tumors, even though they are not encouraging in this respect.

SUMMARY

The influence of birch ash and of a commercial mineral mixture on the growth and transplantability of a malignant tumor in the abdominal cavity of the rat was experimentally investigated. Mixed in the food of the animals these substances could not prevent the growth of the abdominal transplant. The result was also negative in the experiments in which the transplanted tumor was already growing in the abdominal cavity of the animals and in which these substances were injected intraperitoneally.

Acknowledgement. — We wish to thank the Rev. J. Kytömäki, who kindly sent us birch ash. We also thank Professor O. Erämetsä and Miss Marjatta Väänänen, Dipl. Eng., for their spectrographic analysis of the birch ash.

REFERENCES

1. Behan, R. J.: Cancer. Henry Kimpton, London 1938.
2. Bauer, K. H.: Das Krebsproblem. Springer-Verlag, Berlin-Göttingen-Heidelberg 1949.
3. Bullough, N. S.: J. Endocrinol. 1952:*8*:265.
4. Le Long, R. P., Coman, D. R., and Zeidman, I.: Cancer 1950:*3*:718.
5. Teir, H., Voutilainen, A., Kiljunen, A., and Ravanti, K.: Acta Path. et Microbiol. Scand. 1952:*Suppl. 93*:93.
6. Teir, H., Voutilainen, A., and Kiljunen, A.: Acta Path. et Microbiol. Scand. (In press).

FROM THE DEPARTMENT OF FORENSIC MEDICINE, UNIVERSITY OF HELSINKI

STUDIES ON THE TISSUE SPECIFICITY OF RIBONUCLEASE[1]

PRELIMINARY REPORT

by

H. TEIR and T. KUUSI

(Received for publication January 14, 1953)

It has been proved in our laboratory (4) that a suspension of the orbital gland of the rat, when injected into the abdominal cavity, causes a considerable increase in the mitotic ratio of the orbital gland. Since this mitotic stimulator withstands heating (5) it is obviously not a protein. It would appear that it is of the nature of a nucleic acid. We know that ribonucleic acid (RNA) is probably tissue specific, while desoxyribonucleic acid (DNA) is species specific (2). A possible explanation for the organ specificity of the mitotic stimulator would be the hypothesis that the specific RNA in the tissue suspension is influenced by the corresponding ribonuclease in a homologous organ. In order to find out whether tissue specificity in the action of ribonuclease can be established, the following experiments were performed.

METHODS

The effect of ribonuclease was investigated by Brachet's method, using for an object sections of the orbital gland and of liver tissue. The tissue specimens were fixed in Zenker's and Serra's solutions. For staining, pyronine-methyl green was used. Some of the specimens were left untreated for controls, and some were treated with

[1] An investigation aided by a grant from the Sigrid Jusélius Foundation and President J. K. Paasikivi's Fund for Cancer Research.

a raw ribonuclease preparation derived from orbital gland or from liver tissue or with commercial crystalline ribonuclease (Worthington). The degree of staining was measured by Photovolt photometer.

Immediately after decapitation of 1-month-old rats the tissue was cooled and homogenized in a glass homogenizer, after which distilled water was added. Since ribonuclease withstands heating to 100° C. for 5 minutes (3) the rest of the enzymes of the suspension were inactivated by this means. The suspension was then quickly cooled with ice water and was centrifuged. The supernatant liquid was used as the enzyme preparation.

Experiment. — 5 μ thick sections were mounted on slides, and after removal of the paraffin were treated with ribonuclease preparations at 37° C. for 2 hours and 40 minutes. Both the orbital gland and the liver preparations were dealt with in five different ways:

1) untreated controls
2) distilled water
3) orbital gland ribonuclease
4) liver ribonuclease
5) crystalline ribonuclease

RESULTS

By means of photometry the following absorption values were obtained for the different groups:

	Orbital gland			*Liver*		
	Control	Section	Difference	Control	Section	Difference
Controls	53	23	29	51	41	10
Distilled water	52	25	27	52	43	9
Orbital gland ribonuclease	52	43	9	52	42	10
Liver ribonuclease ..	53	45	8	53	47	6
Crystalline ribonuclease	53	49	4	53	50	3

Figure 1 is a diagrammatic representation of the results given in the table. Treatment with distilled water left an almost equal staining as in the untreated controls. Crystalline ribonuclease had

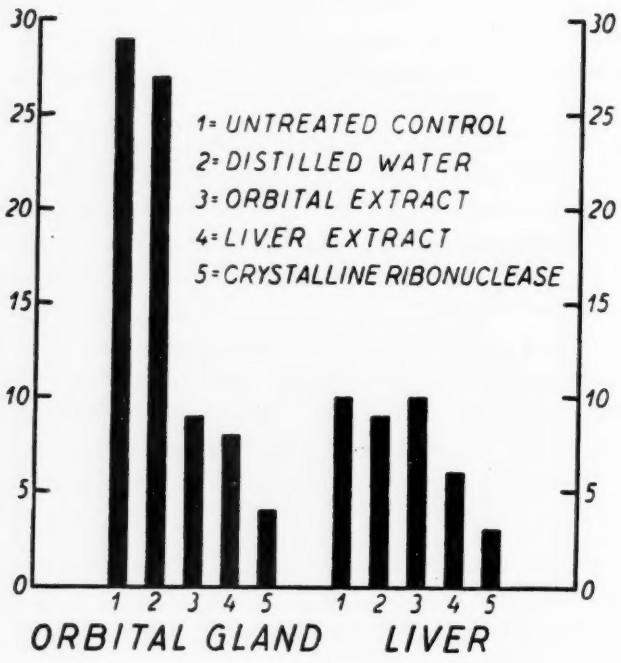

Fig. 1.

destroyed the entire plasmabasophilia and the nuclei only were stained. As to the effect of the tissue suspensions it could be established that in the orbital gland sections plasmabasophilia had decreased considerably through the influence of both the orbital gland and the liver preparation. On the other hand the basophilia of the liver sections was decreased only by the liver preparation, while the orbital gland preparation proved ineffective.

The result thus obtained justifies the assumption that the orbital gland ribonuclease is specific and affects only orbital gland RNA, while the range of effect of the liver ribonuclease is larger.

Investigations for the further elucidation of this problem are continued.

Acknowledgement. — We wish to thank Professor Osmo Järvi of Turku University for his kindness in letting us use the photometer belonging to his Institute.

REFERENCES

1. Brachet, J.: Enzymologia 1951:*10:*87.
2. Chargaff, E.: Experientia 1950:*6:*201.
3. Dubos, R. J., & Thompson, R. H. S.: J. Biol. Chem. 1937:*124:*501.
4. Teir, H.: Acta Pathol. et Microbiol. Scand. 1952:*30:*158.
5. Teir, H.: Growth 1952:*16:*85.

FROM THE DEPARTMENT OF PHYSIOLOGY, UNIVERSITY OF HELSINKI

A NEW METHOD FOR OBTAINING CONTRAST IN LIGHT MICROSCOPY

by

ALVAR WILSKA

(Received for publication January 15, 1953)

In an ordinary light microscope, details of transparent objects with varying refractivity, e.g. the majority of unstained biological specimens cannot be observed due to the lack of contrast if full condenser and objective apertures are used. Under these circumstances, reduction of the apertures is beneficial to the contrast, but it is unavoidably followed by a reduction in the resolving power of the microscope. Oblique illumination may sometimes give better results, but the picture is more-or-less distorted due to the lack of central symmetry in the contrasts thus created. Dark-field microscope makes use of the diffracted rays only and enables us to see objects with sharp discontinuities very brilliantly, whereas those with gradually changing optical density are not observed at all. Phase-contrast microscope of Zernike is definitely superior in this respect. It makes use of a phase-shifting annular area in the objective, closely covering the image of a corresponding ring-shaped aperture in the condenser. In most of the commercial phase-contrast microscopes, the phase plate exerts a retarding influence upon the diffracted rays and causes a carefully predetermined diminution in intensity of the rays passing directly through the field of oservation. The result of both these effects is an image in which objects with greater thickness or optical density appear darker than the surrounding area. The contrast thus obtained is called positive phase contrast. If the phase retardation and absorp-

tion properties of the different parts of the objective aperture are reversed, negative phase contrast is obtained. The former of these methods is used almost exclusively.

Although the phase contrast appears to be relatively simple when applied to a two-dimensional cross-section drawing of a lens system, it becomes very complicated if we put it into a three-dimensional model with apertures as wide as those utilized in high-class microscope objectives. It may take another ten or twenty years before our knowledge of phase effects in microscopical observation is satisfactory. From a report on a recent optical conference (1) one is likely to come to the same conclusion.

When assessing the merits of the methods to be presented here and the part the author of this article has played in their development, there may be reason to know that the author is against much theorizing, favoring empirism when possible. Having the impression that conventional phase-contrast objectives do not utilize more than a limited area in the middle of the total aperture and having tried to apply some kind of »schlieren» method to microscopy, he has made hundreds of experiments with different kinds of condenser and objective apertures, with conditions varying one at a time, in constant search for optimal results. In the course of these experiments, more emphasis has been given to the absorption and form of the apertures than to the phase retardation. This is due greatly to the plainness of means available to the author for the treatment of objective lens surfaces. The only practical way to obtain fairly uniform coatings is to soot the lens surfaces by short contacts with a candle flame until the soot layer reaches the absorption desired. On a watchmaker's lathe, using a sharp tip of some soft material, e.g. cellophane, as a tool, narrow ring-shaped areas, either soot-covered or naked, could be made with little difficulty. It was very fortunate that after each trial the soot formations could be wiped off instantly. During the early part of the experiments, conducted since 1944, it became clear that there were two ways to be chosen. The first of them gives a »positive» and the second a »negative» picture.

Method 1. — To make objects with higher refractivity appear dark against a light background, it is necessary to locate the objective lens surface that comes nearest to the image of the condenser aperture. This surface is furnished with a soot layer thin enough

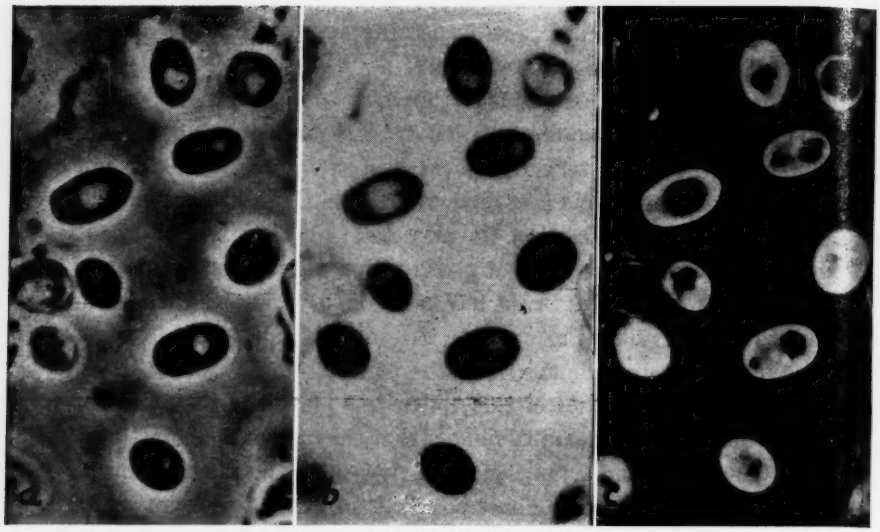

Fig. 1. — Living yeast cells as seen by phase contrast (a), method 1 (b), and method 2 (c). Note the absence of halo effects in b and c and an appreciable gradation of contrasts of different cells in b.

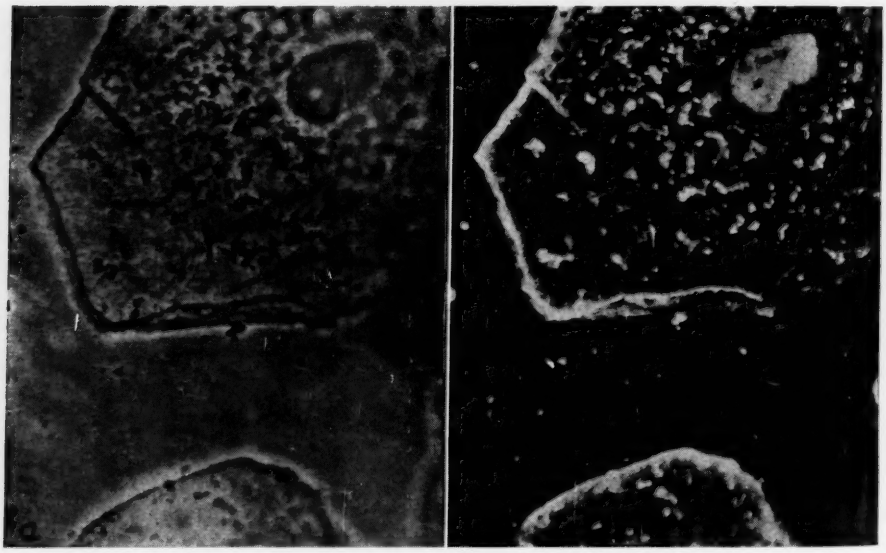

Fig. 2. — A very thin epithelial cell from oral mucosa photographed by phase contrast (a) and mthod 2 (b). The light and shadow effects near the margins of the cell in the latter picture give an illusion of sky-illumination.

to absorb not more than one-half of the light falling on it. The absorption rate of 50% serves only as a guide and is by no means critical. Next comes the question of the width and the diameter of the annulus which is turned on the lathe into the soot layer. Best results are obtained by using a narrow annular naked area with a diameter somewhat more than one-half of the total aperture diameter available. This is at least twice as wide as the phase ring in conventional phase contrast objectives with the same magnification. The annular ring-shaped area in the condenser must be measured accordingly.

In a microscope objective built by this principle, the area covering the image of the condenser ring aperture is unshadowed. Thus, the direct rays coming through the specimen are not influenced in intensity or phase. The diffracted rays, on the other hand, lose about half their intensity before being able to form the final image. Except for their inherent difference in phase compared to the direct rays, there may be some further retardation in the soot layer, although the amount of the latter is undetermined.

Compared with an ordinary phase contrast, method 1 gives weaker contrasts in very thin details. On the other hand, it possesses a higher resolving power, relative freedom from halo effects, and gives a pleasing gradation of objects having different optical densities (Fig. 1). When dealing with highly refracting objects, e.g. yeast cells, spores, pollen, epidermal cells, hairs and sperm heads, these properties are of a definite advantage.

Method 2. — Images of very great contrast are obtained if a partly reversed procedure is followed. The sooting is continued until the transparency of the lens surface is decreased to about ten percent of the original value. Subsequently, the soot is turned off from the surface except for an area covering the image of the condenser ring aperture. The resulting image has light values almost opposite to those produced by method 1 or the conventional phase contrast. One would possibly regard this as being a disadvantage, although this is in reality the mode of illumination we are best accustomed to in everyday life. Ordinary objects in our macroscopical world seldom appear as shadows against a light background. On the contrary, the things themselves are often stronger light-emitting than the background. The sky in daylight, the most natural source of illumination, causes shadows to appear in the vicinity

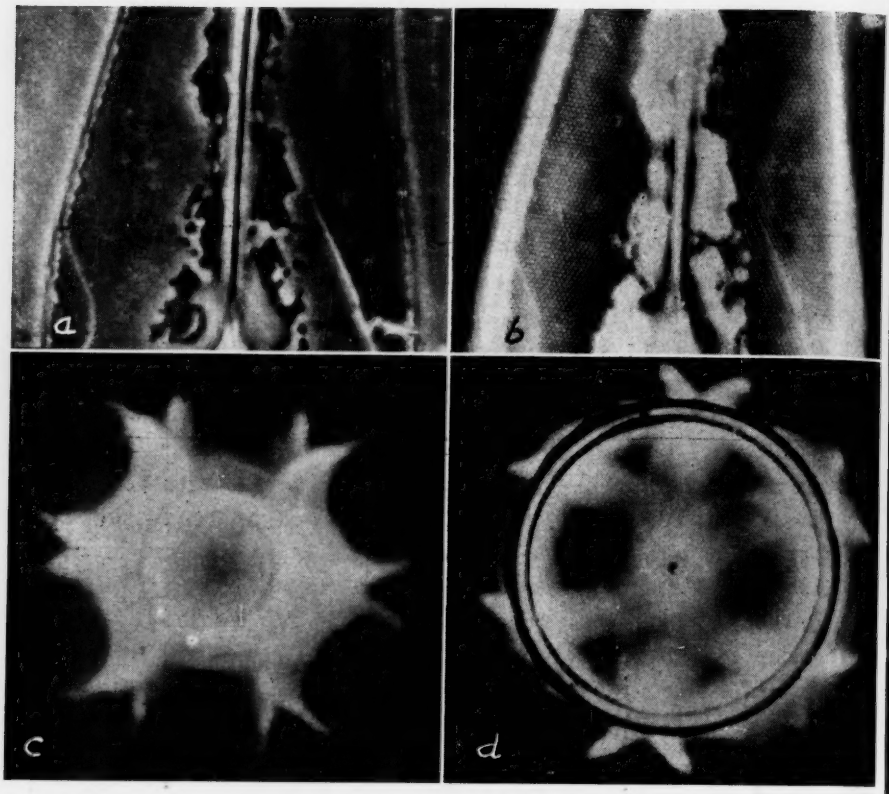

Fig. 3. — Pleurosigma angulatum as seen by phase contrast Reichert 45× (a) and by the corresponding objective modified according to method 2 (b). The structural pattern is not revealed by the phase contrast whereas in b it is clearly visible. Diffraction images c (phase contrast) and d (method 2) show the differences in the size of the aperture rings and corresponding location of the first maxima.

and around the objects instead of the bright halo effects of the positive phase contrast. In fact, method 2 gives surprisingly natural light and shadow effects. If we look at Fig. 2, showing fresh unstained epithelial cells from the oral mucosa, we have an illusion of sky-illuminated giant mollusks lying on the sand floor of a shallow sea. This illusion is strengthened by the agreeable sepia tone in which the whole image inherently appears.

To compare the resolving power of our system with the phase contrast, we have to select a test object having suitable spacings in it's repeating pattern of details. The diatom Pleurosigma angu-

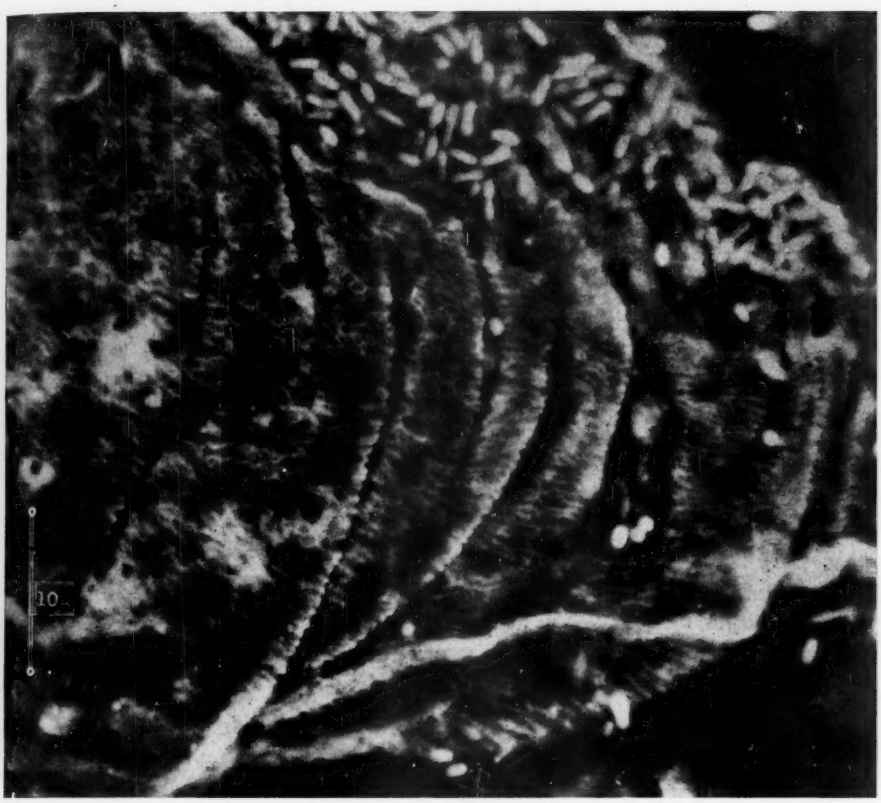

Fig. 4. — The surface structure of an unstained oral epithelial cell. Due to the high magnification only a part of the cell surface is within the picture. Some epiphytic bacteria are also seen.

latum has been frequently used for this purpose. Using an ordinary phase contrast objective 45×, aperture 0.65, absolutely nothing is seen of the fine structure of these algae (Fig. 3 a). Having the same objective (made by Reichert in Austria) furnished by the principle 1 or 2 of the author, the structure becomes clearly visible (Fig. 3 b). In each case, the diffraction images in the objective aperture are radically different. This is shown by Fig. 3 c and d, which also show the difference in size of the aperture rings between the phase contrast objective and that of the author.

The increase in contrast and resolving power is bound to give better images than those previously obtained. It has been proved

that many light-microscopically hitherto more-o-less unseen features are easily rendered visible. Some examples are mentioned. Unstained influenza B virus particles can be seen, especially when attached to red cell ghosts in the usual way. Flagella of many living bacteria are observed very clearly. Around living thrombocytes, spreading membrane margins are easily discernible. Delicate membranous structures are seen in most types of mucus. The minimum thickness of a membrane to be observed needs not exceed 1/100,000 mm.

The author hopes that the present publication will encourage the microscope manufacturers to try to improve the performance of the light microscope further. With better facilities at their disposal, they are not bound to use sooted surfaces on their lenses. But even if the level where we stand were not appreciably raised in the near future, we have come closer to some interesting problems just behind the border of visibility of previous light microscopes. The author is well familiar with the special advantages of the electron microscope. It has a very serious handicap in the necessity of desiccation of the specimen. To see real life and motion in natural environment with greater distinction than before must be our goal for centuries to come.

SUMMARY

Two methods of converting an ordinary microscope into one giving higher resolving power and better contrast than conventional phase contrast microscopes are presented. The annular apertures in the objectives are made in a layer of candle soot.

REFERENCES

1. Proceedings of the London Conference on Optical Instruments, p. 131. Chapman & Hall, Ltd., London 1951.

Note Added in Proof. — The first communication on this subject (A. Wilska; Nature 1953, *171*, 353) was followed by a comment of Dr. R. Barer and the author's reply (Nature 1953, *171*, 697).

ÜBER DAS VORKOMMEN VON CHROMOTROPER SUBSTANZ IM BINDEGEWEBE UND EPITHEL BEI FIBROEPITHELIALEN UND EPITHELIALEN GESCHWÜLSTEN DER WEIBLICHEN MILCHDRÜSE SOWIE BEI CHRONISCHER MASTOPATHIE

von

CLAËS v. NUMERS

(Am 19. 1. 1953 bei der Schriftleitung eingegangen)

Die Kenntnis von den im Organismus vorkommenden Substanzen, die früher schlechthin »Schleim« oder »Mucin« genannt wurden, hat eine vollständige Umwälzung erfahren, seit Lison im Jahre 1935 nachwies, dass diese Substanzen, die mit gewissen Farbstoffen metachromatische Färburg ergeben, zum grössten Teil aus Esterschwefelsäuren mit hohem Molekulargewicht bestehen. Auf diese Untersuchung folgten die Arbeiten von Jorpes und Mitarbeiter, durch welche klargelegt wurde, dass das Heparin eine Mucoitin-Polyesterschwefelsäure ist, sowie die Entdeckung von Holmgren und Wilander, dass die Granula der Ehrlichschen Mastzellen aus Heparin bestehen. Dank der fortgesetzten Untersuchungen der letztgenannten Forscher wurde die Kenntnis von den sowohl frei im Gewebe als auch gebunden — in Form von granulahaltigen Mastzellen — vorkommenden chromotropen Substanzen erweitert. Dieselben liessen sich vor allem in wachsenden, mesenchymalen Geweben, im Knorpel und Knochen sowie in gewissen epithelialen Elementen nachweisen.

Wie die oben genannten Autoren benutzte auch Sylvén bei seinen zahlreichen Untersuchungen die Färbung mit Toluidinblau. Mit diesem Farbstoff geben die chromotropen Substanzen — unter der Voraussetzung einer richtigen Technik — eine spezifische, echte metachromatische Farbreaktion. Sylvén konstatierte u.a. das Vorkommen von chromotroper Substanz im Granulations-

gewebe (1942) sowie in solchen Teilen verschiedener mesenchymaler Geschwülste, die im Wachstum begriffen sind (1945).

In einer im Jahre 1940 publizierten Arbeit über das Vorkommen von hochmolekularen Esterschwefelsäuren in epithelialen Tumoren gibt Sylvén folgende Erläuterungen:

Ruhende epitheliale Strukturen weisen keine Metachromasie im umgebenden Stroma auf. Bei Epithelvegetationen, die im Wachsen begriffen sind, wie z.B. Fibroadenomatose der Milchdrüse, kommt im jungen Stroma eine mässige Menge metachromatischer Substanz vor, welche an Quantität zunimmt, wenn es sich um einen infiltrativ wachsenden Epitheltumor handelt. Bei ausgesprochen leuko- oder lymphozytärer Infiltration sowie nach Strahlenbehandlung nimmt die metachromatische Färbbarkeit des Stromas entweder ab oder verschwindet.

In einer später (1949) erschienenen Arbeit hat Sylvén weitere Berichte geliefert über das durch Epithelwachstum hervorgerufene Auftreten von Polysaccharid-Estersulfate. Dieselben kamen sowohl bei gutartigen als malignen Epithelproliferationen im Stromabindegewebe und in dem interzellulären Medium vor, desgleichen, wenn schon in erheblich kleineren Mengen, auch im Zytoplasma der Epithelzellen. Gemäss Sylvéns Untersuchungen waren bei langsam wachsenden Karzinomen in dem umgebenden Bindegewebe deutliche degenerative Veränderungen, grosse Mengen chromotroper Substanzen und ausgeprägte Wucherungstendenz wahrzunehmen. Bei Fällen von rapide wachsendem Karzinom ging auch der degenerative Prozess im Bindegewebe schnell vor sich, die metachromatischen Substanzen kamen nur spärlich vor und die Wucherungstendenz war niedrig oder fehlte gänzlich.

Es schien mir der Mühe wert, epitheliale und fibroepitheliale Geschwülste der weiblichen Milchdrüse sowie Fälle von chronischer Mastopathie auf das Vorkommen von chromotropen Substanzen im Bindegewebe und deren eventuelles Vorkommen im Epithel näher zu untersuchen. Wie aus dem oben Gesagten hervorgeht, hat sich das Auftreten der genannten Substanzen frei im Bindegewebe als charakteristisch für wachsende Gewebe erwiesen. Eine Untersuchung von der hier in Frage stehenden Art könnte somit einen Beitrag zur Kenntnis von der Aktivität und Zuwachsintensität der Gewebe bei verschiedenen pathologischen Zuständen der Milchdrüse liefern.

Ausser den oben dargelegten Mitteilungen in Sylvéns Arbeiten habe ich über das Thema, das ich hier zur Behandlung genommen habe, in der Literatur keine anderen Angaben angetroffen als diejenigen von Jääskeläinen (1951). In seiner Arbeit war jedoch die männliche Milchdrüse Gegenstand der Untersuchung. Von 20 Karzinomfällen fand Jääskeläinen nur in zwei reichlich freie metachromatische Substanz oder Mastzellen im Bindegewebe, in den übrigen 18 Fällen nur spärlich oder überhaupt nicht. In Fällen von Gynäkomastie schienen die Verhältnisse ist dieser Hinsicht variabler zu sein, da der Verfasser bei einem grossen Teil der untersuchten Drüsen reichlich freie chromotrope Substanz und Mastzellen im sog. Mantelbindegewebe feststellten konnte.

Für die Untersuchung von metachromatischer Substanz eignet sich die von Holmberg und Wilander ausgearbeitete Methode mit Fixierung in einer 4%igen Lösung von basischem Bleiazetat am besten. Wie Sylvén gezeigt hat, tut indessen auch die Fixierung in gewöhnlicher Formalinlösung die gleichen Dienste, vorausgesetzt dass die Fixierungszeit nicht 48 Stunden überschreitet. Da mir formalinfixiertes Material zur Verfügung stand, habe ich von demselben bei meinen Untersuchungen Gebrauch gemacht. Bei der Auslese der Fälle wurde die Fixierungszeit kontrolliert. Dieselbe belief sich im allgemeinen auf rund 24 Stunden. Die Färbung wurde in einer 0.5%igen Lösung von Toluidinblau in destilliertem Wasser ausgeführt. Nach der Färbung folgte Entwässerung in 96%igem und absolutem Alkohol, um die Entstehung falscher metachromatischer Reaktionen zu verhindern.

Auf die oben beschriebene Weise sind insgesamt 17 Fälle von pathologischen Veränderungen in der Milchdrüse untersucht worden. Im Folgenden werden die bisher gemachten Beobachtungen dargelegt.

Laktierende Milchdrüse. — Eines von den zur folgenden Gruppe gehörenden Fibroadenomen wurde einer stillenden Frau entnommen, die drei Monate früher ein Kind geboren hatte. Da in dem Präparat auch Partien von normalem Milchdrüsengewebe enthalten waren, bot sich die Gelegenheit, das Verhalten der Milchdrüse während der Stillperiode zu untersuchen. Die Struktur der Drüse war die für das Funktionsstadium typische. Die Alveolen waren gross, ausgedehnt, sehr dicht nebeneinander liegend, nur durch äusserst schmale Bindegewebssepten voneinander getrennt.

In den Epithelzellen waren deutliche Zeichen der Absonderungstätigkeit.

Weder in den Epithelzellen noch im Sekret konnten metachromatisch gefärbte Substanzen wahrgenommen werden. Auch die schmalen Zwischenwände der Alveolen waren frei von chromotropem Stoff. Nur in den grössten Interlobularsepten wurde stellenweise eine sehr schwache positive Färbung erzielt, die sich hauptsächlich in der Umgebung der Blutgefässe lokalisierte. Mastzellen vom gewöhnlichen Typus wurden spärlich im interlobularen Bindegewebe angetroffen, auch diese meistenteils perivaskulär.

Fibroadenoma mammae. — Vier Fälle von typisch gebauten perikanalikularen Fibroadenomen wurden untersucht. Das derbe, grobfaserige, zellarme Bindegewebe, das den Hauptteil dieser Geschwülste ausmacht, verhielt sich in sämtlichen Fällen negative, d.h. metachromatische Färbung konnte nirgends nachgewiesen werden. Mastzellen, die von gewöhnlichem Aussehen und reich an Granula waren, lagen in mässiger Anzahl in dem Gewebe eingestreut.

Die spärlichen Drüsengänge waren von typischem, einfachem Bau, das Epithel niedrig. Nur in der aus einer laktierenden Milchdrüse (vgl. oben) stammenden Geschwulst war das Epithel leicht proliferierend und wies Anzeichen sekretorischer Tätigkeit auf. Metachromatisch färbbare Substanzen waren nirgends im Epithel anzutreffen.

Das zellreichere, in einer schmalen Schicht die Drüsengänge umgebende Bindegewebe, das dem Mantelbindegewebe entspricht, war in zwei Fällen durchgehend frei von chromotropen Substanzen. In den zwei anderen Fällen konnte an vereinzelten Stellen eine äusserst schwache metachromatische Reaktion konstatiert werden. Mastzellen kamen in diesem Mantelbindegewebe praktisch genommen überhaupt nicht vor. Nur in einem Falle konnten hier einige vereinzelte Mastzellen wahrgenommen werden.

Mastopathia chronica. — Von den 5 hierzu zählenden Fällen stellte eine die leichteste (früheste?) Form dar, die von manchen Forschern als Mazoplasie bezeichnet wird (vgl. Kuzma und Ingleby). Das Drüsenelement kam in Form von kleinen, unregelmässigen Lobuli mit teilweise schwach dilatierten Gängen vor. Das Epithel war ruhig, nicht proliferierend. In demselben waren keine metachromatischen Substanzen vorhanden. Das Mantelbindegewebe war

spärlich und von dem umgebenden Stützbindegewebe unscharf abgegrenzt, enthielt keine chromotropen Stoffe, Mastzellen dagegen in mässiger Anzahl. In dem Stützbindegewebe konnte nirgends positive Färbung beobachtet werden. Mastzellen kamen in etwa derselben Menge wie in dem Mantelbindegewebe vor.

Die folgenden zwei Fällen von chronischer Mastopathie gehören zunächst zu der Form, die als Adenose bezeichnet wird, und für welche grosse, unregelmässige Drüsenlobuli und vermehrtes interlobuläres Bindegewebe sowie Zystenbildungen variabler Grösse kennzeichnend sind. In den von mir untersuchten Fällen war das Epithel in den meisten Gängen etwas proliferierend, an manchen Stellen geschichtet, das Plasma der Epithelzellen oft vakuolisiert. Hinsichtlich metachromatischer Färbbarkeit verhielt sich das Epithel durchaus negativ.

Mantelbindegewebe war reichlich vorhanden, in beiden Fällen wies es ziemlich starke Metachromasie und Mastzellen in mässiger Zahl auf.

Das Stützbindegewebe war in beiden Fällen von gewöhnlichem Aussehen, es fehlte gänzlich an freier chromotroper Substanz.

Zystische Mastopathie. — Die voll ausgebildete, zystische Form der Mastopathie war durch 3 Fälle vertreten. Von diesen waren 2 durch mässige Epithelproliferation und typische Zystenbildungen ausgezeichnet, der dritte durch hochgradig proliferierendes Drüsenepithel, welches an mehreren Stellen solide Stränge bildete. In diesem letztgenannten Fall sowie in einem von den früher erwähnten wurde in solchen Partien, wo Zeichen apokriner Sekretion vorkamen, beobachtet, dass die ins Gang- oder Zystenlumen hineinragenden Sekretkuppeln deutliche metachromatische Färbung gaben. Ebenso verhielt es sich an diesen Stellen mit dem in der Drüse oder in den Zysten angesammelten Sekret. Die grosse Mehrzahl der Drüsen verhielt sich indessen auch in diesem Fällen negativ.

In einem von den drei Fallen war das Mantelbindegewebe schwach entwickelt und erwies sich als arm sowohl an freier chromotroper Substanz als auch an Mastzellen. In den beiden anderen Fällen wurden wechselnde, stellenweise starke Metachromasie und verhältnismässig zahlreiche Mastzellen im Mantelbindegewebe festgestellt.

Das Stützbindegewebe war in allen drei Fällen frei von chromotropen Substanzen.

Karzinom der Milchdrüse. — Insgesamt wurden sieben Fälle von verschiedenartigen Milchdrüsenkarzinomen der Untersuchung auf chromotrope Substanzen unterzogen. Wir werden hier die Resultate dieser Untersuchung von Fall zu Fall der Reihe nach besprechen.

Bei einem nicht infiltrierenden Milchgangskarzinom mit aus anaplastischem Epithel aufgebauten soliden und semisoliden Kolben und Strängen vermisste man alle Zeichen von Metachromasie im Epithel. Auch die in vielen Epithelfeldern durch zentrale Erweichung entstandene Detritusmasse verhielt sich überall negativ. Das lockere und recht zellreiche Stromagewebe, das zunächst die Epithelstränge umgab, war im allgemeinen stark metachromatisch und enthielt mässig bis reichlich Mastzellen von gewöhnlichem Aussehen. Es kamen jedoch da und dort Epithelgänge vor, deren Mantelbindegewebe nicht das geringste Zeichen von metachromatischer Reaktion aufwies, obwohl sich keine histologischen Unterschiede zwischen dem Epithel in diesen und demjenigen in den anderen Gän en feststellen liessen.

Das vom Epithel ferner gelegene, mehr zellarme Bindegewebe war im allgemeinen frei von chromotroper Substanz.

In dem Präparat von diesem Falle wurden an einigen Stellen Drüsenlobuli mit etwas proliferierendem aber nicht karzinomatösem Epithel angetroffen. Ihr Mantelbindegewebe war schwach metachromatisch und wies spärlich Mastzellen auf.

In einem Fall von infiltrativ wachsendem Karzinom, das seinem morphologischem Typus gemäss als ein Milchgangkrebs aufzufassen war, gab das Epithel stellenweise eine ganz schwache, das Stroma hingegen überall eine kräftige metachromatische Reaktion. Auch in solchen Abschnitten der Geschwulst, wo nur ganz vereinzelte Karzinomzellen im Bindegewebe vorkamen, hatte dieses einen kräftigen violetten Farbton angenommen.

Zwei niedrig differenzierte Adenokarzinome mit ausgedehnten soliden, markigen Geschwulstbezirken wiesen ein von chromotroper Substanz freies Epithel auf, während das Stroma überall positiv, wennschon nicht besonders stark gefärbt war. Mastzellen kamen im Stroma reichlich vor. Dieselben wiesen jedoch einen von den typischen Mastzellen abweichenden Bau auf, indem die Granula bedeutend kleiner und im allgemeinen heller waren, mehr rötlich gefärbt als gewöhnlich. In dem ausserhalb des eigentlichen Geschwulstgebietes liegenden Bindegewebe wurden zahlreiche Mastzellen vom gewöhn-

lichen Typus wahrgenommen. Der Unterschied zwischen diesen und den in der Geschwulst vorkommenden war beträchtlich.

In einem alveolär gebauten, teilweise zirrhösen Karzinom sowie in einer rein zirrhösen Geschwulst wurde starke Metachromasie im Stroma sowie zahlreiche Mastzellen vom gleichen »Tumortypus» wie in den beiden vorhergehenden Fällen festgestellt. Das Epithel verhielt sich überall negativ.

Der letzte Tumor in dieser Serie war ein stark anaplastischer Gallertkrebs. Er drehte sich um einen grossen, inoperablen Brustdrüsenkrebs, der sich nach intensivster Strahlenbehandlung nebst grossen fortwährenden Testosterongaben erheblich verkleinert hatte. Drei Wochen nach beendeter radiologischer Behandlung wurde bei der Patientin eine palliative Operation durchgeführt, der die untersuchten Präparate entstammen.

Das Geschwulstparenchym bestand aus unregelmässigen Haufen von atypischen, stark vakuolisierten Epithelzellen. Stromagewebe war reichlich vorhanden. In den Maschen desselben kamen grosse Massen einer amorphen, schleimigen Substanz vor. Die meisten von den in den Krebszellen erscheinenden Vakuolen hatten keine Färbung angenommen. Bei einer grossen Anzahl Zellen wurden jedoch metachromatisch gefärbte Tropfen wahrgenommen, die von wechselnder Grösse, meistens aber beträchtlich kleiner als die ungefärbten Vakuolen waren. Die oben erwähnten schleimartigen Massen waren ungefärbt oder höchstens äusserst schwach, kaum merkbar blau gefärbt. Das Stromagewebe selbst hatte sich kräftig metachromatisch gefärbt. Mastzellen konnten im Tumorgewebe nirgends entdeckt werden.

Geht man von der Voraussetzung aus, dass, wie es Sylvén (1942) dargelegt hat, die Intensität des metachromatischen Farbtons bzw. die Anzahl der Mastzellen eine Schätzung der ungefähren Menge von chromotroper Substanz ermöglichen, können die vorliegenden Beobachtungen als Urteil über die Quantität der jeweiligen Gewebsvermehrung verwendet werden. Im grossen und ganzen sind die Resultate mit früheren Erfahrungen und mit den primären Vermutungen übereinstimmend.

Das Vorkommen von metachromatischen Substanzen im Epithel der Milchdrüse in grösseren Mengen war a priori kaum zu erwarten. In der Tat hat es sich auch in dieser Hinsicht hauptsächlich negativ

verhalten. Bei Fällen von voll ausgebildeter Mastopathie scheinen jedoch metachromatische Stoffe in den Epithelzellen auftreten zu können, ausnahmsweise auch bei Karzinomfällen. Ob die in dem zuletzt dargelegten Karzinomfall beobachteten violettgefärbten Granula eventuell als eine Folge der Strahlenbehandlung entstanden waren, entzieht sich meiner Beurteilung.

Weit bedeutungsvoller ist natürlich die Frage über das Vorkommen von chromotropen Substanzen im Bindegewebe. Dass das Stroma der laktierenden Milchdrüse eine schwache Metachromasie in einer Periode, während welcher keine Weiterentwicklung der Drüse stattfinden sollte, aufwies, kann vielleicht als ein Zeichen für die relativ lebhafte Neubildung von Epithel, die auf die Drüsenfunktion folgt, aufgefasst werden.

In den Fibroadenomen konnten keine metachromatischen Substanzen oder nur Spuren derselben entdeckt werden. Dies steht in gutem Einklang mit dem anerkannt langsamen Zuwachs dieser Geschwülste.

Bezüglich der Verhältnisse bei chronischer Mastopathie kann man feststellen, dass die metachromatische Reaktion des Mantelbindegewebes in quantitativer Hinsicht in grossen Zügen der Intensität der Epithelproliferation entspricht. Eingeschaltete Perioden von relativer Ruhe im pathologischen Geschehen sowie anfängliche regressive Prozesse können unter Umständen scheinbare Regelwidrigkeiten veranlassen.

Sämtliche untersuchten Fälle von Karzinom waren durch deutliche, meistens starke Metachromasie im Stroma charakterisiert. Zu bemerken ist jedoch, dass karzinomatöse Gebiete mit schwacher oder fehlender metachromatischer Stromareaktion vorkommen, ohne dass spezielle degenerative Zeichen im Epithel wahrzunehmen waren. Das Verhalten der Mastzellen im Karzinomgewebe war insofern bemerkenswert, als die Granula derselben kleiner waren und sich heller färbten als die der in anderen Geweben vorkommenden. Vielleicht steht diese Tatsache in Beziehung zu einer gesteigerten Heparinabgabe.

Ein Sachverhalt, auf welchen ich aufmerksam machen möchte, ist die Auffallend kräftige metachromatische Reaktion des Stromagewebes in den zirrhösen Karzinomen. Durch diese Beobachtung wird noch besonders die stark aktive Teilnahme des Bindegewebes an dem Wachstumsprozess in diesen Fällen hervorgehoben. Die

erhebliche Wucherung und die starke Metachromasie des Bindegewebes sowie der vergleichsmässig geringfügige epitheliale Einschlag deuten auf ganz besondere Verhältnisse in den Beziehungen zwischen Epithel und Bindegewebe. Man muss sich fragen, ob dieses Verhalten etwa im Zusammenhang stehe mit Erfahrungen, die eine wachstumshemmende Wirkung der chromotropen Substanz auf das Epithel vermuten lassen.

Zum Schluss einige Worte über den präoperativ radiologisch behandelten Fall von Gallertkrebs. Die Abwesenheit von Mastzellen im Tumorgewebe stimmt mit Sylvéns Beobachtungen über die strahlenbehandelten Geschwülste überein. Im schroffen Gegensatz dazu steht aber das reichliche Vorkommen von freier chromotroper Substanz im Stromagewebe. Die in diesem Falle vorhandenen reichlichen Gallertmassen haben augenscheinlich keine hochmolekularen Esterschwefelsäuren enthalten, da sie keine metachromatische Färbung angenommen hatten.

ZUSAMMENFASSUNG

Der Verfasser hat 17 Fälle von pathologisch veränderten Milchdrüsen sowie funktionierendes Milchdrüsengewebe aus der Stillperiode auf chromotrope Substanzen untersucht. In zwei Fällen von chronischer Mastopathie und in einem strahlenbehandelten Karzinom kamen spärlich metachromatische Substanzen in den Epithelzellen vor. Bezüglich des Bindegewebes wurden folgende Resultate erzielt:

Laktierende Milchdrüse: Schwache Metachromasie.

Fibroadenoma: Metachromasie fehlend oder sehr schwach.

Chronische Mastopathie: Negative bis starke Reaktion, im allgemeinen der Proliferation des Epithels entsprechend.

Karzinom: Mässige bis starke Reaktion, besonders stark in zirrhösen Geschwülsten.

SCHRIFTTUM

HOLMGREN, HJ.: Zschr. mikr.-anat. Forsch. 1940:*40:*488.
HOLMGREN, HJ., u. WILANDER, O.: Ibid. 1937:*42:*242.
JORPES, E., u. Mitarb.: Zit. nach SYLVÉN 1942.
JÄÄSKELÄINEN, V.: Diss. Helsinki 1951.
KUZMA, J., u. HELEN INGLEBY: The breast. Anderson, W. A. D.: Pathology. Mosby Company, St. Louis 1948.
LISON, L.: Zit. nach SYLVÉN 1942.
SYLVÉN, B.: Nord. Med. 1940:*5:*115.
SYLVÉN, B.: Acta Chir. Scand. 1941:*86:* Suppl. 65.
SYLVÉN, B.: Acta Radiolog. Suppl. 1945:59.
SYLVÉN, B.: Acta Radiolog. 1949:*32:*11.

FROM THE DEPARTMENT OF HYGIENE, UNIVERSITY OF HELSINKI, AND
FROM THE PHARMACOLOGICAL LABORATORY, ORION LTD., HELSINKI

EFFICIENCY OF CERTAIN DISINFECTANTS AS SERUM PRESERVATIVES

by

EINO V. VENHO and ILONA VENHO

(Received for publication January 28, 1953)

The efficiency of most disinfectants is largely dependent on the conditions under which they are used. There is no method at our disposal capable of determining and expressing the relative efficiency of disinfectants under all possible conditions, and no method available enables us to take into consideration all the variable factors that are involved in practice.

Rideal and Walker (18) developed a unit, known as the phenol coefficient, which makes it possible to compare the bactericidal properties of different substances. As disinfectants of different types have greatly divergent mechanisms of action, the phenol coefficient has in many cases proved to be unsatisfactory in practice (16, 11). It is a suitable measure for comparing aromatic compounds related to phenol, but in comparing other substances it often gives entirely misleading results (8). When testing e.g. sublimate, its phenol coefficient is found to be about 14 if the bacteria are exposed for 2 ½ minutes, which should be sufficient to kill them, while an exposure of 30 minutes gives a phenol coefficient of about 550 (13). This is due to the fact that phenol acts rapidly, but sublimate slowly. When employed in sublethal concentrations, many disinfectant agents can inhibit the growth of microbes which may still be able to cause infection if introduced into living tissues. A good disinfectant should, however, be capable of destroying the microbes.

Since the factors influencing the efficiency of disinfectants are so various and commercial interest frequently leads to the publication of the most favourable results alone, the efficiency of every disinfectant should be tested under conditions similar to those of actual use.

The aim of our study was to examine substances of different types which have been or could perhaps be employed as preservatives for blood plasma, sera, vaccines, etc.

We have studied the bacteriostatic and the bactericidal action of phenol, tricresol, chlorocresol, formaldehyde solution, merthiolate, methyl para-hydroxybenzoate and propyl para-hydroxybenzoate in broth and horse serum.

METHOD

Our aim was to arrange the test conditions so as to be at least as favourable to bacteria as those obtaining in practice. The disinfectant under examination was allowed to act on the bacteria for 2 to 3 days because an earlier use of sera and corresponding preparations is very rare. The temperature was kept at the optimum level for bacterial growth.

A series of dilutions of the substances under investigation were placed in test-tubes containing broth or serum, and the media were then inoculated with the different bacterial suspensions, mould or sewage. The bacterial suspensions had been obtained by cultivating them in broth. These strains had been secured from clinical routine investigations carried out at the Institute of Serology and Bacteriology, University of Helsinki. The same strains were used throughout the investigation. Mould had been taken from an infected Petri dish and cultivated at room temperature, at which it had shown good growth. Sewage had been obtained from the sewage works, where a sample with the highest possible bacterium content had been chosen.

The media, to which serial dilutions of the disinfectant under investigation had been added, were inoculated with 2 to 3 drops of the bacterial suspensions. The tubes were then placed in a thermostat and incubated at 37° C for 2 to 3 days. Mould cultures were kept at room temperature, or about 20° C. Sewage was added

to the media by means of a pipette: 0.5 ml to each tube containing about 10 ml of the medium.

Two to 3 days later the cultures were examined for the lowest concentration that had exerted a bacteriostatic action. From such tubes 0.5 ml of the culture was transferred into a sterile medium by means of a pipette, which made the dilution of the disinfectant concerned 18 to 20 times weaker. Thus the conditions should have been favourable to the growth of possibly viable microbes. If no growth appeared within 3 to 4 days, the disinfectant was regarded as having had a bactericidal action, and the lowest bactericidal concentration was accordingly recorded.

PHENOL

In 1867 Lister introduced phenol, or carbolic acid, as an antiseptic for use in surgery. Substances better suited for surgical purposes have since been discovered, but phenol still retains an important place in some fields of modern medicine. Phenol has a characteristic odour and is soluble in 13 to 14 parts of water.

Phenol has been included in the present study because it is used as a measure of bactericidal efficiency. Phenol is a common protoplasmic poison, the action of which is as highly injurious to bacteria as to other living cells and, in general opinion, more physical than chemical (17). Phenols react with bacterial proteins to form insoluble albuminates. Their toxicity is due to their ability to denature proteins. A parallelism between the denaturation of protein and the bactericidal action has been noted (2). Other investigations have led to the conclusion that the phenols kill by interference with the oxidation-reduction potential equilibrium of the cells (20). Rapidity of action is characteristic of phenol. Pus, feces and other organic matter reduce its efficiency but little, on the average 10 per cent. Fungi and viruses are considerably more resistant to phenol than bacteria (11).

For practical disinfection large amounts of phenol are required in fairly strong concentrations, which often irritate the tissues. A 2 per cent solution has been used for washing hands and a 5 per cent solution for sterilising instruments.

Several factories employ phenol as a preservative for vaccines and certain other preparations, of which only small quantities are

TABLE 1

LOWEST BACTERIOSTATIC CONCENTRATION OF DISINFECTANT FOR VARIOUS MICRO-ORGANISMS IN BROTH

Micro-organism	Phenol	Tricresol	Chlorocresol	Formal-dehyde	Merthiolate	Methyl p-hydroxy-benzoate	Propyl p-hydroxy-benzoate
Escherichia coli	1: 500	1: 700	1: 9,000	1: 20,000	1: 400,000	1: 1,000 +	1: 3,000
Staphylococcus aureus	1: 400	1: 800	1: 5,000	1: 15,000	1: 300,000	1: 1,000 +	1: 2,000
Streptococcus pyogenes	1: 600	1: 1,000	1: 4,000	1: 20,000	1: 200,000	1: 1,000	1: 2,000
Pseudomonas pyocyanei	1: 400	1: 1,000	1: 2,000	1: 10,000	1: 90,000	1: 1,000 +	1: 2,000 +
Mould	1: 600	1: 1,100	1: 6,000	1: 15,000	1: 100,000	1: 1,000 +	1: 2,000
Sewage	1: 300	1: 500	1: 3,000	1: 10,000	1: 100,000	1: 1,000 +	1: 2,000 +

+ Indicates bacterial growth.

TABLE 2

LOWEST BACTERICIDAL CONCENTRATION OF DISINFECTANT FOR VARIOUS MICRO-ORGANISMS IN BROTH

Micro-organism	Phenol	Tricresol	Chlorocresol	Formal-dehyde	Merthiolate	Methyl p-hydroxy-benzoate	Propyl p-hydroxy-benzoate
Escherichia coli	1: 320	1: 600	1: 7,000	1: 15,000	1: 300,000	1: 1,000 +	1: 2,000 +
Staphylococcus aureus	1: 300	1: 400	1: 3,000	1: 15,000	1: 300,000	1: 1,000 +	1: 2,000 +
Streptococcus pyogenes	1: 600	1: 900	1: 4,000	1: 20,000	1: 200,000	1: 1,000	1: 2,000
Pseudomonas pyocyanei	1: 400	1: 600	1: 2,000	1: 10,000	1: 90,000	1: 1,000 +	1: 2,000 +
Mould	1: 300	1: 1,000	1: 6,000	1: 15,000	1: 80,000	1: 1,000 +	1: 2,000 +
Sewage	1: 200	1: 400	1: 3,000	1: 10,000	1: 300,000	1: 1,000 +	1: 2,000 +

+ Indicates bacterial growth.

TABLE 3

+ Indicates bacterial growth.

TABLE 3

LOWEST BACTERIOSTATIC CONCENTRATION OF DISINFECTANT FOR VARIOUS MICRO-ORGANISMS IN HORSE SERUM

Micro-organism	Phenol	Tricresol	Chlorocresol	Formal-dehyde	Merthiolate	Methyl p-hydroxy-benzoate	Propyl p-hydroxy-benzoate
Escherichia coli	1: 400	1: 700	1: 2,000	1: 20,000	1: 12,000	Saturated solution —	Saturated solution +
Staphylococcus aureus	1: 200	1: 400	1: 1,500	1: 9,000	1: 12,000	» +	» +
Streptococcus pyogenes	1: 300	1: 500	1: 4,000	1: 30,000	1: 18,000	» —	» +
Pseudomonas pyocyanei	1: 300	1: 400	1: 1,500	1: 10,000	1: 2,000	» +	» +
Mould	1: 400	1: 700	1: 3,000	1: 10,000	1: 10,000	» —	» +
Sewage	1: 300	1: 600	1: 1,500	1: 10,000	1: 2,000	» +	» +

+ Indicates bacterial growth.

TABLE 4

LOWEST BACTERICIDAL CONCENTRATION OF DISINFECTANT FOR VARIOUS MICRO-ORGANISMS IN HORSE SERUM

Micro-organism	Phenol	Tricresol	Chlorocresol	Formal-dehyde	Merthiolate	Methyl p-hydroxy-benzoate	Propyl p-hydroxy-benzoate
Escherichia coli	1: 400	1: 600	1: 1,500	1: 10,000	1: 10,000	Saturated solution +	Saturated solution +
Staphylococcus aureus	1: 200	1: 200	1: 1,500 +	1: 9,000	1: 12,000	» +	» +
Streptococcus pyogenes	1: 300	1: 400	1: 2,000	1: 20,000	1: 18,000	» +	» +
Pseudomonas pyocyanei	1: 300	1: 400	1: 1,500 +	1: 10,000	1: 1,000	» +	» +
Mould	1: 400	1: 700	1: 3,000	1: 10,000	1: 10,000	» +	» +
Sewage	1: 200	1: 500	1: 1,500	1: 10,000	1: 1,000	» +	» +

+ Indicates bacterial growth.

administered by intramuscular or subcutaneous injection. The phenol concentration most commonly used for these purposes is 0.5 per cent (3).

In the present investigation phenol was found to exert a bacteriostatic action in dilutions varying from 1: 300 to 1: 600 when the organisms were suspended in broth (Table 1), while somewhat stronger concentrations, i.e. from 1: 200 to 1: 600, were generally required to kill the bacteria (Table 2).

In many cases the bacteriostatic and bactericidal concentrations were found to be stronger in horse serum than in broth. The dilutions used in horse serum cultures varied from 1: 200 to 1: 400. In no case was it necessary to use a stronger concentration than 1: 200, which corresponds with the phenol concentration employed by many factories in the preservation of vaccines and other preparations.

TRICRESOL

Tricresol is a viscous liquid having a characteristic odour. When preserved, it turns reddish brown on exposure to air (oxygen) and light. It is a mixture of three isomeric compounds, viz. ortho-, meta- and para-cresol. These three isomeric substances differ little in their antiseptic properties and are commonly used as a mixture, tricresol, which is considerably cheaper than its pure components. Commercial tricresol is more or less impure, often containing phenol, fatty acids, oils and other impurities which may greatly affect its bactericidal efficiency. Its solubility is rather poor, one part of tricresol dissolving in about 50 to 60 parts of water. The phenol coefficient of tricresol is about 2½ to 3. Its toxicity is a little lower than that of phenol, while its mode of action as well as its resorptive properties and local effects are similar to those of phenol. The cresols are effective against most of the common bacteria including acid-fast bacteria.

The presence of organic matter does not greatly reduce the efficiency of tricresol (12, 11). It is less effective against viruses; its virucidal powers are, however, stronger than those of phenol. It has been employed as a preservative for vaccines, etc., most commonly at a concentration of 0.25 per cent.

The tricresol used in our experiments had been manufactured by Schering-Kahlbaum A.G., Berlin.

Our experiments showed that concentrations of tricresol varying from 1: 500 to 1: 1100 were strong enough to exert a bacteriostatic action on the different microbes in broth cultures (Table 1), while concentrations of 1: 400 to 1: 1000 had a bactericidal action (Table 2). In horse serum, concentrations of 1: 400 to 1: 700 inactivated the bacteria (Table 3) and concentrations of 1: 200 to 1: 700 killed them (Table 4).

On examining the results, attention is drawn to the fact that tricresol did not prove to be much more effective than phenol. In most cases its efficiency was 1½ to 2 times as great as that of phenol, but against some microbes the same.

CHLOROCRESOL

Chlorocresol, or parachlormetacresol, occurs as colourless crystals with a characteristic phenolic odour. It is soluble in water, at 20° C in the proportion of 1:250, and very soluble in alcohol and ether. Its phenol coefficient is 30.5 and its toxic effects are slight. Different organic substances considerably lower its efficiency (7). It is used e.g. in the preservation of various cosmetic preparations, where it has proved to be better than para-hydroxybenzoic acid. Proportions of 0.05 to 0.1 per cent of chlorocresol have been regarded as sufficient to preserve cosmetic preparations and perfumes have been added to efface the possible odour (6). One per cent of chlorocresol has been added to various soaps used as general disinfectants.

Many pharmaceutical preparations contain 0.2 per cent of chlorocresol as a preservative. In some countries there are statutory regulations enforcing the addition of chlorocresol to different medicines administered by injection, such as procaine, adrenalin and bismuth preparations (3).

In our experiments we found that the bacteriostatic efficiency of chlorocresol against different microbes considerably varied in broth cultures. The most resistant microbe was *Pseudomonas pyocyanei*, whose growth was inhibited by chlorocresol in a concentration of 1: 2000, the efficiency of chlorocresol being 5 times greater than that of phenol. A concentration of 1: 9000 was sufficient to inactivate *Escherichia coli*, which showed an efficiency 17 times stronger than that of phenol (Table 1).

The concentrations of chlorocresol required to produce a bactericidal action on certain bacteria in broth were somewhat stronger than those necessary for bacteriostatic action. The requisite dilutions varied from 1: 2000 to 1: 7000 (Table 2).

To produce a bacteriostatic action, concentrations of chlorocresol 2 to 4 times stronger were required in horse serum than in broth (Table 3). The higher protein content of horse serum evidently impeded the action of chlorocresol. In serum the action of chlorocresol was on the average 5 times stronger than that of phenol, although its phenol coefficient is as high as 30.5.

In horse serum the quantities of chlorocresol needed to kill the bacteria had to be considerably larger than those required to inactivate them. It was found that the concentration 1: 1500 was not in every case capable of exerting a bactericidal action (Table 4). The action of chlorocresol on even the most sensitive microbes was not more than 7.5 times stronger than that of phenol, and its action on the more resistant microbes was only three times stronger than that of phenol under corresponding conditions. The efficiency of chlorocresol in horse serum was not as much as one-tenth of that expressed by its phenol coefficient.

FORMALDEHYDE

Commercial formaldehyde solution usually contains 37 to 41 per cent of formaldehyde. Formaldehyde solution is a colourless liquid with an intense pungent odour.

Solution of formaldehyde has a high bactericidal and virucidal efficiency. Its action is due to the fact that formaldehyde reacts with the amino groups of proteins to form chemical compounds. The reaction takes place slowly, and the dilutions commonly employed do not cause precipitation of the protein (21, 11). The presence of organic matter dos not greatly reduce the efficiency of formaldehyde solution (12).

Formaldehyde solution has been extensively used as a preservative for various cosmetic preparations and tooth-pastes, but this practice is now on the decline. Formaldehyde solution is, however, still used in the preservation of different foods and milk. It is also used in the treatment of toxins to produce toxoids.

In our experiments formaldehyde proved to be effective in very dilute solutions, its bactericidal concentrations being but slightly stronger than those required to give a bacteriostatic action (Tables 1—4). The presence of organic matter did not lower its efficiency to any appreciable degree, but its germicidal activity was almost the same in horse serum as in broth.

In broth formaldehyde retained its efficiency in dilutions of 1: 10000 to 1: 20000, showing germicidal powers 25 to 50 times as strong as those of phenol. Dilutions of approximately the same strength were needed in horse serum, but the efficiency of formaldehyde, as compared to that of phenol under the same conditions, was somewhat greater.

MERTHIOLATE

Merthiolate[1], or sodium ethyl mercuri thiosalicylate, occurs as a light-cream-coloured crystalline powder containing about 49 per cent of mercury in organic combination. The substance is readily soluble in water. Merthiolate cannot be used with acids, the salts of heavy metals or iodides. Its toxicity is fairly low. It has been reported that rabbits tolerate intravenous injections of 20 to 25 mg of merthiolate per 1 kg of body weight. Human subjects have also been given merthiolate in a 1 per cent solution intravenously, the maximum single dose being 50 cc, without serious subsidiary effects (31).

The phenol coefficient of merthiolate has been determined by several investigators, the lowest values being 40 to 50 and the highest about 200 (10). Like many other organic mercurial compounds, merthiolate is more effective but considerably less irritating than mercuric chloride. Merthiolate exerts *in vitro* a strong bacteriostatic action, which may be due to the slow freeing of mercury from its organic compound. Merthiolate does not cause precipitation of serum proteins, not even in strong concentrations.

Merthiolate has been used fairly extensively as a preservative for biological preparations. In a dilution of 1: 10 000 it has been found to kill various bacterial suspensions without changing their antigenic properties. It has been reported that diphtheria toxoid

[1] Merthiolate is the trade-mark of Eli Lilly and Company for its brand of sodium ethyl mercuri thiosalicylate.

preserved with merthiolate retains its immunising property better than does the same toxoid preserved with phenol (15, 5). According to the investigations of Strumia *et al.* (22), merthiolate in the proportion of 1: 10 000 should be added to plasma if it is to be kept more than one day before being used. It is a common practice to use a stock solution containing 1 per cent of merthiolate, which is prepared by dissolving 1 g of merthiolate and 1.4 g of borax in 100 ml of distilled water. One ml of this stock solution should be added to each 100 ml of the liquid to be preserved.

Our experiments call attention to the exceedingly strong bacteriostatic action that merthiolate has in broth. Merthiolate was effective against different microbes in dilutions of 1: 90 000 to 1: 400 000. Its bactericidal activity was hardly less pronounced.

In horse serum the efficiency of merthiolate was found to be only a fraction of what it was in broth. Bacteriostasis was produced in horse serum by dilutions ranging from 1: 2000 to 1: 18 000, while somewhat stronger concentrations were required to kill the bacteria.

PROPYL PARA-HYDROXYBENZOATE

Propyl para-hydroxybenzoate occurs as a white crystalline powder, which is soluble in water, 1: 2000.

Back (1) found propyl para-hydroxybenzoate to be effective against yeasts. Eschenbrenner's (4) investigations led him to recommend the use of 0.04 to 0.1 per cent of propyl para-hydroxybenzoate as an effective preservative for eye drops containing various alkaloids. The average strength of the solutions employed has been about 0.05 per cent. The substance is not, in general, an irritant, and there is no evidence to suggest at present that it possesses any sensitising properties (9).

Our experiments showed that dilutions of 1: 2000 were capable of exerting a bacteriostatic action on some bacteria in broth, while they were ineffective against others (Table 1). This concentration was found to have a bactericidal action only on the strain of streptococci employed, whereas the other bacteria grew well (Table 2).

In most cases, saturated solution in serum, with undissolved particles of the substance remaining at the bottom of the test tubes, did not appear to be able to retard bacterial growth, much less to inhibit it.

METHYL PARA-HYDROXYBENZOATE

Methyl para-hydroxybenzoate occurs in the form of white crystals or as a white powder, which is soluble in water in the proportion of about 1: 600. It is of very low toxicity and hardly irritates the tissues in the concentrations ordinarily employed. It has been used as a preservative for many pharmaceutical preparations, such as syrups and infusions. For such purposes, a concentration of 0.1 to 0.2 per cent has been regarded as suitable. Methyl para-hydroxybenzoate has been used as a preservative also for various eye drops because it has no appreciable action on the conjunctiva (19). Since it is practically harmless and not a primary irritant, it has also been employed for the sterile preservation of injection fluids (9).

In our experiments we found that a 1: 1000 dilution of methyl para-hydroxybenzoate failed to prevent bacterial growth in broth and to give any protection against the bacteria (Tables 1 and 2).

We also tested the protective qualities of a saturated solution in horse serum but found that, even with a layer of undissolved methyl para-hydroxybenzoate at the bottom of the test tube, bacterial growth continued practically uninhibited (Tables 3 and 4). In our experiments the bacteria grew as if there had been no preservative.

DISCUSSION

We have carried out investigations into the efficiency of certain disinfectants of various types in broth and horse serum. Such disinfectants were selected as have been or may be used for the preservation of plasma, sera, vaccines, etc.

Phenol was included in our study mainly to provide a standard of comparison. The experiments showed that in no case was it necessary to use a concentration stronger than 1: 200, which corresponds with the phenol concentration employed by several factories in the preservation of vaccines, etc. According to many previous investigations, this concentration is incapable of destroying fungi and viruses (11).

In our experiments tricresol proved to be less effective than previously estimated. Although its phenol coefficient has been

recorded as being 2½ to 3, we found that its efficiency against most micro-organisms was only 1½ to 2 times that of phenol and against certain micro-organisms the same. It is evident that the serum protein impedes the action of tricresol to a greater degree than do the suspensions of human feces employed as organic matter in most other investigations. It is clear that as a preservative 0.25 per cent of tricresol in the substance to be preserved does not always give sufficient protection against different microbes.

The efficiency of chlorocresol was not always found to be as much as one-tenth of its phenol coefficient. It is evident that the serum proteins considerably interfere with the action of chlorocresol.

The efficiency of formaldehyde solution was almost as great in broth as in serum, being 25 to 50 times that of phenol under corresponding conditions.

The efficiency of merthiolate was very considerably less in horse serum than in broth. Our investigations clearly show that, as a preservative e.g. for plasma, merthiolate in a dilution of 1: 10 000 gives incomplete protection and that other precautionary measures and sterility must in no case be neglected. According to previous researches, mercurial preparations in the concentrations ordinarily employed are incapable of destroying bacterial spores.

On the basis of our experiments it is evident that methyl para-hydroxybenzoate and propyl para-hydroxybenzoate cannot inhibit bacterial growth if the conditions, i.e. the medium and the temperature, are suitable for it.

SUMMARY

The aim of our investigation was to study substances of different types which have been or which might be used as preservatives for plasma, sera, vaccines, etc. We found that in a concentration of 1: 200 phenol was capable of killing all the microbes we employed; therefore the protection given by this phenol concentration can be regarded as good. Tricresol proved to be less effective than its phenol coefficient implies, and a dilution of 1: 400 does not afford sufficient protection against bacteria.

In our experiments the efficiencies of chlorocresol and merthiolate were weak. Methyl para-hydroxybenzoate and propyl para-hydroxybenzoate failed to give any protection against bacteria in serum.

The efficiency of formaldehyde solution proved to be the best and steadiest in our experiments, being almost equally high in serum as in broth.

It is clear that the high protein content of serum seriously interferes with the action of numerous disinfectants, the result of which is that the phenol coefficient is entirely misleading. As preservatives for sera and plasma the substances under investigation, in the concentrations used at present, give only feeble protection.

REFERENCES

1. Back: Pharm. J. 1932:*128:*328 vide Harry (6).
2. Cooper and Woodhouse: Biochem. J. 1923:*17:*600, vide McCulloch (11).
3. Dilling, W. J.: The Pharmacology and Therapeutics of Materia Medica, Cassell and Company, London, 1951.
4. Eschenbrenner, H.: Apoth. Ztg. 1932:*47:*630.
5. Frazer, J. N.: Am. J. Technol. 1942:*8:*89.
6. Harry, R. G.: Cosmetic Materials. Vol. II., Leonard Hill Limited, London, 1948.
7. Klarmann, E., Shternov, V. A., and von Wowern, J.: J. Bakt. 1929: *17:*423.
8. Leonard, G. F.: J. Infect. Dis. 1931:*48:*358.
9. Leschke, E.: München med. Wchnschr. 1930:*77:*2006.
10. Marshall, M. S.: California & West. Med. 1931:*35:*43.
11. McCulloch, E. C.: Disinfection and Sterilization, Second Ed., Lea & Febiger, Philadelphia, 1945.
12. McCulloch, E. C., and Costigan, S.: J. Infect. Dis. 1936:*59:*281.
13. Møller, K. O.: Farmakologi, Nyt Nordisk Forlag. Arnold Busck, Copenhagen, 1952.
14. Powell, H. M., and Jamieson, W. A.: Am. J. Hyg. 1931:*13:*296.
15. Powell, H. M., and Jamieson, W. A.: Proc. Indiana Acad. Sci. 1938: *47:*65.
16. Reddish, G. F.: Ind. and Eng. Chem. 1937:*29:*1044 1 vide McCulloch (11).
17. Reichel, H.: Biochem. Ztschr. 1909:*22:*149.
18. Rideal, S., and Walker, J. T. A.: Jour. Roy. Sanitary Inst. 1903: *24:*424, vide McCulloch (11).
19. Salmony: La Parfumerie Moderne 1932:*26:*607 vide Harry (6).
20. Schubert, K., and Richter, K.: Zentralbl.f. Bakt. 1926:*66:*11.
21. Sollmann, T.: A Manual of Pharmacology, Saunders Company, Philadelphia and London, 1949.
22. Strumia, M. M., Wagner, J. A., and Monagham, J. F.: Ann. Surg. 1940:*111:*623.

AUS DEM PHARMAKOLOGISCHEN INSTITUT DER UNIVERSITÄT HELSINKI

VERGLEICHENDE VERSUCHE ÜBER DIE WIRKUNG VERSCHIEDENER ATMUNGSSTIMULANSEN

I Teil

von

EINO V. VENHO, OSMO VARTIAINEN und MATTI VAPAAVUORI

(Am 28. 1. 1953 bei der Schriftleitung eingegangen.)

Das Zentralnervensystem stimulierende Stoffe gibt es eine ganze Menge, und sie weichen bezüglich ihrer chemischen Struktur, ihres Wirkungsmechanismus' und ihrer Wirkungsstelle beträchtlich voneinander ab. Die meisten Stimulansen des Zentralnervensystems beeinflussen gleichzeitig mehrere Zentren, wie z.B. das Atmungs- und das vasomotorische Zentrum. Ihre eigene, beschränktere Gruppe bilden von den Stimulansen des Zentralnervensystems die zentralen Analeptika oder Wiederbelebungsmittel, welche die obengenannten lebenswichtigen Zentren stimulieren, und welche eine Weckwirkung aus der Narkose besitzen. Mit den zentralen Analeptika erzielt man oft pharmakologisch geradezu dramatisch erscheinende Wirkungen. In therapeutischem Sinne kommt diesen Stoffen nur eine begrenzte Bedeutung zu. Es ist ziemlich unmöglich, die Stimulierung des Zentralnervensystems längere Zeit fortzusetzen. Je kräftiger die Stimulation gewesen ist, umso schneller stellen sich Verminderung der Aktivität des Zentralnervensystems nach der Stimulierung sowie Abnahme der Intensität und der Dauer einer neuen Stimulation ein. Alle diese Umstände begrenzen den Gebrauch der zentralen Analeptika auf eine kurze Zeit und in erster Linie auf akute Fälle. In diesen Fällen ist ihre Wirkung allerdings oft lebensrettend. Derartige Indikationen sind u.a. Fälle von Überdosierung narkotischer Stoffe, schwere Schlafmittelvergiftungen, Ertrinkungs- und Elektrizitätsunfälle, verschiedene

akute Atmungs- und Kreislaufkollapse zentraler Herkunft sowie gewisse Stadien der Infektionskrankheiten.

Untersuchungen über die Stimulansen des Zentralnervensystems sind recht zahlreich ausgeführt worden. Da der Wirkungsmechanismus, die Art, Stärke und Dauer der Wirkung sehr voneinander abweichen, ist es natürlich, dass die Stoffe sich schwer miteinander vergleichen lassen, und dass es schwierig ist, ein geeignetes Versuchsobjekt für diesen Zweck zu finden. Die Resultate mit verschiedenen Versuchstieren stimmen nicht überein und können nicht ohne weiteres auf die klinische Medizin angewandt werden. Ausserdem haben die zentralen Analeptika einen verschiedenen antagonistischen Wert gegen jeden einzelnen das Zentralnervensystem deprimierenden Stoff, so dass die bezüglich eines deprimierenden Stoffes durchgeführten Versuche nicht immer besagen, dass die Wirkung in Bezug auf einen anderen deprimierenden Stoff die gleiche wäre, und ausserdem haben auch noch die angewandten Dosen grosse Bedeutung.

In unseren eigenen Untersuchungen haben wir die Wirkung verschiedener Atmungsstimulansen auf die durch Morphin herabgesetzte Atmung beim Kaninchen ausprobiert. Gegenstand der Untersuchung waren im ersten Teil unserer Untersuchungen das Strychnin, Pikrotoxin, Lobelin, Koffein und 1-Phenyl-2-Methylaminopropan (Pervitin).

DIE UNTERSUCHUNGSMETHODE

Bei den Atmungsversuchen benutzten wir als Versuchstiere ausgewachsene Kaninchen, denen als atmungsherabsetzender Stoff 4 mg/kg Morphin hydrochlorid intravenös verabreicht wurde, welche Dosis die normale Atmung des Kaninchens, die gewöhnlich ca. 2 ½—6 Liter in fünf Minuten betrug, auf ein Viertel oder die Hälfte des Normalen herabsetzte. Zur Messung des Atmungsvolumens benutzten wir den auf Abb. 1 gezeigten Apparat. Um die Maske dicht am Kopf des Versuchstiers anlegen zu können, wurden an den Stellen, wo die Maske am Kopf anlag, die Haare mit Vaselin eingefettet. Beim Beginn des Versuchs liessen wir das Kaninchen zuerst eine zeitlang sich an den Apparat gewöhnen und sich beruhigen, wonach das Volumen der vom Versuchstier verbrauchten Luft in Serien von je fünf Minuten gemessen wurde. Die zu unter-

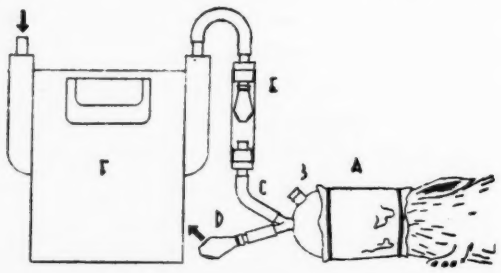

Abb. 1. — Apparat zur Messung des Atmungsvolumens bei Kaninchen. A. Maske des Kaninchens. B. Kork. C. Gummischlauch. D. Exspirationsklappe. E. Inspirationsklappe. F. Gasmesser.

suchenden Stoffe wurden danach in die Vene injiziert. Die Atmungsfrequenz, die beim gleichen Kaninchen in den verschiedenen Versuchen konstant war, und bei den verschiedenen Individuen stark variierte, indem sie 25—100 Mal pro eine halbe Minute betrug, wurde durch den Einfluss des Morphins durchschnittlich auf die Hälfte des Normalen und bisweilen noch mehr reduziert.

DAS STRYCHNIN

Die Wirkung des Strychnins betrifft in erster Linie das Rückenmark und erst bei grossen Dosen beeinflusst es den Rückenmarkfortsatz und die Hirnrinde. Das Strychnin erhöht die Irritabilität aller Reflexbahnen und verstärkt u.a. diejenigen Reflexe, die über den Rückenmarkfortsatz die Kreislauf- und Atmungsfunktionen regulieren. Die Beschleunigung und Vertiefung der Atmung sind die ersten Symptome der Strychninvergiftung, die sich vor den Krämpfen wahrnehmen lassen. Travell und Gold (21) sind der Ansicht, dass die durch grosse Strychnindosen verursachte Atmungslähmung indirekt von der Ermüdung des Atmungszentrums herrührt. Das Strychnin setzt die Reizungsschwelle herab, so dass alle normalerweise unterhalb des Schwellenwerts liegenden peripheren Impulse fortdauernd das Atmungszentrum beeinflussen können und so die Atmungslähmung herbeiführen.

Wenn die normale Atmung des Kaninchens im Voraus durch Morphin reduziert ist, so wird mit Strychnin Steigerung der Atmungsfrequenz und des Atmungsvolumens erzielt (1). Beim gesunden Menschen rufen subkutan verabreichte Dosen von 1—2 mg

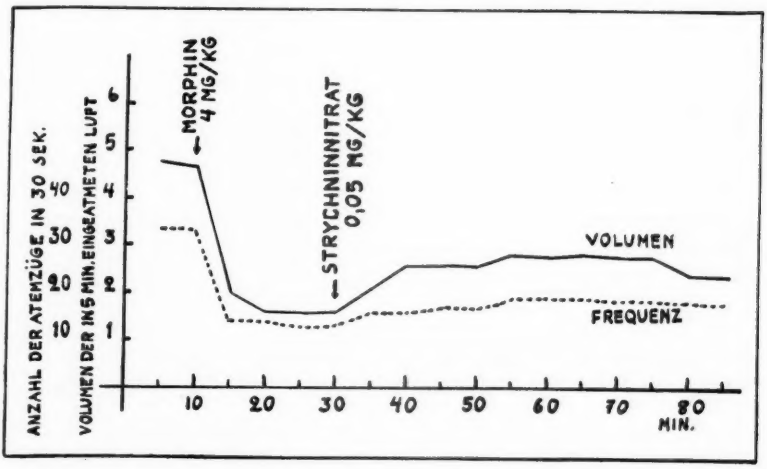

Abb. 2. — Die Wirkung des Strychnins auf die durch Morphin herabgesetzte Atmung des Kaninchens. Gewicht des Tieres 2.5 kg. Morphinhydrochlorid 4 mg/kg intravenös und Strychninnitrat 0.05 mg/kg intravenös.

noch keine Erhöhung der Atmungsfrequenz oder des Atmungsvolumens hervor (15). Mit grösseren Dosen (6 mg) erzielte Lindhard (14) deutliche Beschleunigung der Atmung.

Die Wirkung des Strychnins auf die durch Morphin herabgesetzte Atmung des Kaninchens haben wir in 5 Versuchen untersucht.

Bei Anwendung einer intravenösen Dosis von 0.05 mg/kg Strychninnitrat erzielten wir geringe Zunahme der Atmung (Abb. 2), aber bei keinem Versuch konnte dit Atmung auf das Normale zurückgebracht werden. Die schwache atmungsstimulierende Wirkung des Strychnins dauerte ca. ½—1 Stunde an. Bei Dosen von 0.075 mg/kg und 0.1 mg/kg bekamen die Kaninchen Krämpfe und starben innerhalb von 1—2 Minuten.

DAS PIKROTOXIN

Das Pikrotoxin ist ein seit altersher bekanntes Krampfgift, dessen Wirkung am stärksten den Rückenmarkfortsatz und das Mittelhirn betrifft. Das Pikrotoxin beeinflusst stimulierend das Atmungs-, das vasomotorische und das Brechzentrum. Die therapeutische Breite des Stoffes ist so klein, dass er klinische Anwendung nur in der Behandlung schwerer Barbituratvergiftungen findet, bei

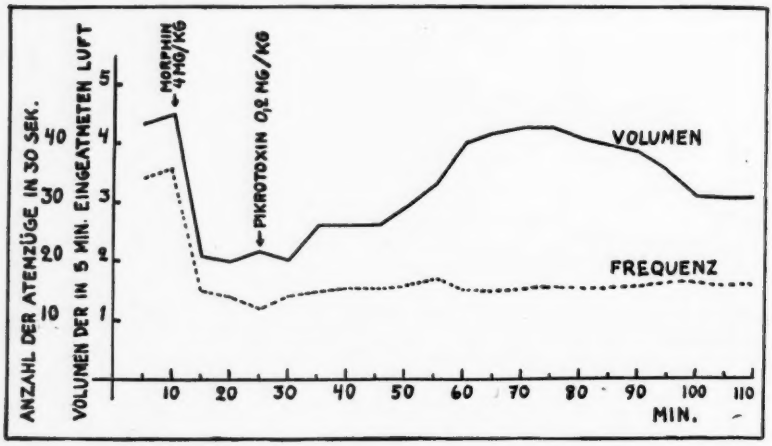

Abb. 3. — Die Wirkung des Pikrotoxins auf die durch Morphin herabgesetzte Atmung des Kaninchens. Gewicht des Tieres 2.85 kg. Morphinhydrochlorid 4 mg/kg intravenös und danach Pikrotoxin 0.2 mg/kg intravenös.

welchen mit den anderen zentralen Analeptika nur schwache oder gar keine Wirkung erzielt wird (16, 12, 11). Mit Pikrotoxin können die Kaninchen nicht aus der mit grossen Morphindosen (50—250 mg/kg) erzeugten Narkose erweckt werden (13, 12). Das Pikrotoxin hemmt in gewissem Masse die vom Morphin verursachte Verminderung der Atmung und des Kreislaufs (12).

Die Wirkung des Pikrotoxins auf die durch Morphin (4 mg/kg intravenös) herabgesetzte Atmung des Kaninchen haben wir in 8 Versuchen untersucht. Die intravenös verabreichten Pikrotoxindosen schwankten von 0.15 bis 0.3 mg/kg.

In zwei Versuchen verabreichten wir den Kaninchen 0.15 mg/kg Pikrotoxin. Bei dem einen Versuchen kehrte die mit Morphin herabgesetzte Atmung auf das Normale zurück. Bei dem anderen Versuch war die Zunahme des Atmungsvolumens nicht so deutlich und erreichte nicht das normale Niveau. Die Atmungsfrequenz nahm unter der Wirkung des Pikrotoxins nur ein wenig zu. Die Versuchstiere waren während des Versuchs ruhig.

In drei Versuchen wurde den Kaninchen 0.2 mg/kg Pikrotoxin verabreicht. Bei allen Versuchen wurde eine deutliche Wirkung erzielt und die Atmung kehrte zum normalen Niveau zurück. Die Atmungsfrequenz nahm nur wenig zu (Abb. 3). Bei dieser Dosis

von 0.2 mg/kg war ein Kaninchen etwas erregt, aber die beiden anderen verhielten sich ruhig.

In zwei Versuchen verabreichten wir den Tieren 0.25 mg/kg Pikrotoxin. Bei diesen Versuchen wurde eine deutliche Wirkung erzielt. Bei dem einen Versuch stieg das Atmungsvolumen nahezu auf das normale Niveau und bei dem anderen sogar darüber hinaus. Die Kaninchen waren etwas unruhig, sie hatten gewissermassen Krampfneigung aber noch keine Krämpfe. Die Wirkung war nicht wesentlich besser als bei der vorherigen kleineren Dosis von 0.2 mg/kg.

Bei einem Versuch bekam das Kaninchen 0.3 mg/kg Pikrotoxin, und die Wirkung war anfangs gut, aber nach 5—10 Minuten begannen die Krämpfe und das Kaninchen starb.

Die Wirkung des Pikrotoxins war nicht plötzlich, sondern nahm ziemlich gleichmässig zu, indem sie nach ca. 15—30 Minuten am stärksten war, um danach wieder langsam abzunehmen. Die Wirkung hielt gewöhnlich 40—90 Minuten lang an.

DAS LOBELIN

Im Jahre 1921 isolierte Wieland (23) das Lobelin, welches dann sofort in der Therapie als Atmungsstimulans Anwendung fand (4). Es wurde beobachtet, dass kleine Dosen die Atmung stimulierten, während grosse zu Lähmung führten und tonisch-klonische Krämpfe verursachten (24).

Nach den späteren Untersuchungen geht die Stimulierung der Atmung über das chemosensible Glomus caroticum, welche das Lobelin stärker anregt als Sauerstoffmangel und Kohlendioxyd, (9, 5). Grössere Lobelindosen und bisweilen schon therapeutische Dosen können schädliche Kreislaufwirkungen herbeiführen, wie Sinken des Blutdrucks und Pulsverlangsamung als Zeichen von Vagusreizung sowie später Anstieg des Blutdrucks (24, 20), sowie Unregelmässigkeit in der Herzfunktion (25). Die Wirkung des Lobelins ist von kurzer Dauer, und seine nachteiligen Kreislaufwirkungen machen es ungeeignet in Fällen, bei denen der Kreislauf gleichzeitig mit der Atmung gestört ist. In diesen Fällen ist den zentralen Analeptika der Vorzug vor dem Lobelin zu geben. Bisweilen ist auch seine Wirkung auf die Atmung weniger günstig

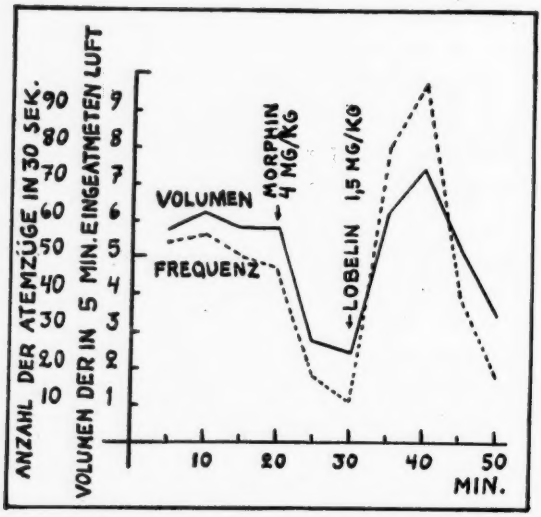

Abb. 4. — Die Wirkung des Lobelins auf die durch Morphin herabgesetzte Atmung des Kaninchens. Gewicht des Tieres 3.2 kg. Morphinhydrochlorid 4 mg/kg intravenös und danach Lobelinhydrochlorid 1.5 mg/kg intravenös.

wegen den von ihm verursachten unregelmässigen Atmungsbewegungen und Hustenanfällen (18).

In unseren eigenen Untersuchungen probierten wir die Wirkung des Lobelins auf die durch Morphin herabgesetzte Atmung des Kaninchens in zehn Versuchen aus. In drei Versuchen wurde intravenös 0.5 mg/kg, 1 mg/kg und $1^1/_2$ mg/kg Lobelin verabreicht. Schon mit einem halben Milligramm wurde ein geringer, aber doch deutlich wahrnehmbarer Effekt von kurzer Dauer erzielt. Ein Milligramm genügte, um das Atmungsvolumen für fünf Minuten bis fast auf das normale Niveau zu steigern. Wenn den Kaninchen intravenös 1.5 mg Lobelin pro kg gegeben wurde, so konnte eine Steigerung des Atmungsvolumens und der Atmungsfrequenz über das Normale erzielt werden (Abb. 4). Während des Versuchs verhielt das Tier sich ruhig.

Bei drei Versuchen verabreichten wir 2 mg/kg Lobelin. In allen diesen Versuchen erreichte die mit Morphin herabgesetzte Atmung ihr normales Niveau, und zweimal stieg das Atmungsvolumen über das Normale hinaus. Bei einem Versuch bekam das Kaninchen für eine kurze Zeit schwache Krämpfe, was indessen bei keinem anderen Versuch vorkam, obwohl wir auch grössere Dosen benutzten. In

der Atmungsfrequenz zeigte sich die entsprechende Zunahme wie auch im Atmungsvolumen. Bei zwei Versuchen bekamen die Tiere 2.5 mg/kg Lobelin intravenös und in zwei weiteren Versuchen 3 mg/kg. Bei diesen Versuchen stieg das Atmungsvolumen ebenfalls auf das normale Niveau oder etwas darüber. Irgendeine deutlichere Wirkung riefen diese grösseren Lobelindosen nicht hervor. Krämpfe traten bei den Tieren nicht auf, aber manche von ihnen zeigten eine gewisse Unruhe. Die Atmungsfrequenz stieg entsprechend wie auch das Volumen. Die Wirkung des Lobelins war in den meisten Versuchen kurz und dauerte meistens nur 5—10 Minuten.

DAS KOFFEIN

In früheren Untersuchungen ist festgestellt worden, dass das Koffein sowohl die Atmungsfrequenz als auch das Atmungsvolumen erhöht (3). Die Wirkung des Koffeins beruht offenbar auf seinem direkten Einfluss auf das Atmungszentrum (17). Es ist beobachtet worden, dass beim Menschen die subkutane Koffeininjektion deutlich den alveolären Druck des Kohlendioxyds herabsetzt und die Atmungsfrequenz erhöht. Auch die durch Morphin oder Heroin herabgesetzte Atmung wird durch das Koffein stimuliert (10).

Die Wirkung des Koffeins auf die durch Morphin herabgesetzte Atmung des Kaninchens haben wir in unsgesamt 11 Versuchen untersucht. Wir benutzten Coffeinum cum natrii bentzoate-Lösung, die wir intravenös in Dosen von 5—75 mg/kg verabreichten.

Mit den kleinsten von uns benutzten Dosen (5 mg/kg, 10 mg/kg, 20 mg/kg und 25 mg/kg) haben wir keine deutliche Wirkung erzielt.

In zwei Versuchen, bei welchen 40 mg/kg Koffein verabreicht wurden, erzielten wir eine geringe Steigerung des Atmungsvolumens, ca. 15—20%, welche ungefähr 10—20 Minuten lang anhielt.

Bei zwei weiteren Versuchen verabreichten wir 50 mg/kg Koffein, wonach sich schon deutliche Steigerung des Atmungsvolumens zeigte, obwohl es noch nicht das normale Ausmass erreichte. Die Wirkung des Koffeins hielt dann ca. 30—40 Minuten lang an, wonach das Atmungsvolumen wiederum abnahm. Das eine Versuchstier war etwas unruhig, aber Krämpfe kamen nicht vor.

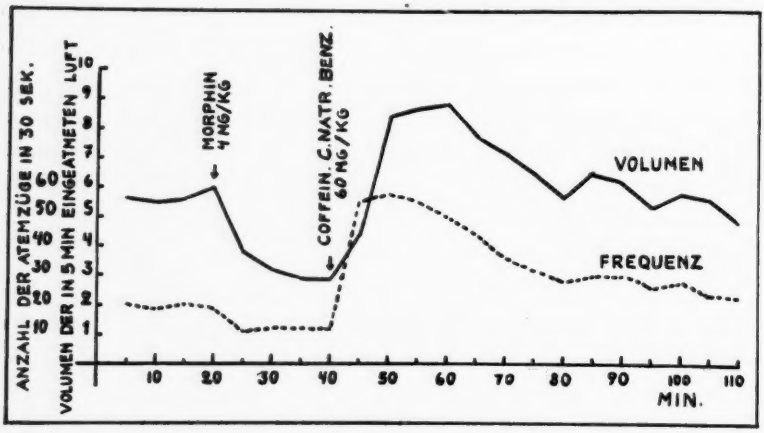

Abb. 5. — Die Wirkung des Koffeins auf die durch Morphin herabgesetzte Atmung des Kaninchens. Gewicht des Tiers 2.6 kg. Morphinhydrochlorid 4 mg/kg intravenös und danach Coffein c. natrii bentsoate-Lösung 60 mg/kg intravenös.

In ebenfalls zwei Versuchen wurde 60 mg/kg Koffein verabreicht. Bei dem einen Versuch bekam das Kaninchen ca. 20 Sekunden dauernde Krämpfe und das Atmungsvolumen wurde bis über das Doppelte vom normalen Ausmass erhöht. Bei dem anderen wiederum war das Tier ruhig, und auch hier stieg das Atmungsvolumen über das Normale und ging nach ungefähr einer Stunde auf das normale Ausmass zurück (Abb. 4).

Wir machten noch zwei Versuche, bei welchen 75 mg/kg Koffein intravenös verabreicht wurde. Beide Kaninchen bekamen starke Krämpfe und das eine starb nach ungefähr einer Minute. Bei dem anderen zeigte sich sehr beträchtliche Zunahme des Atmungsvolumens. Bei allen Versuchen wurde unter der Einwirkung des Koffeins in der Atmungsfrequenz entsprechende Zunahme wie im Atmungsvolumen festgestellt.

DAS PERVITIN

Das Pervitin, 1-Phenyl-2-Methylaminopropan hydrochlorid, wirkt stimulierend auf das Zentralnervensystem ein, und offenbar betrifft seine stimulierende Wirkung auch direkt das Atmungszentrum. Sowohl bei den Menschen als auch bei den Tieren sind sehr grosse individuelle Unterschiede bezüglich der Wirkung des

Pervitins beobachtet worden. Im allgemeinen stimuliert das Pervitin das Atmungszentrum, aber in vielen Publikationen werden Fälle erwähnt, bei denen das Pervitin das Atmungszentrum lähmend beeinflusst hat. Hauschild (7) sah, dass an der Katze in Numalnarkose die durch Morphin gelähmte Atmung durch Pervitin erregt wird, so dass die Atemgrösse zunimmt. Bei Tierversuchen ist festgestellt worden, dass das Pervitin in der Alkoholnarkose antagonistisch wirkt, während es in der Chloralosenarkose umgekehrt die Dauer der Narkose verlängert (8). Blume und Zöller (2) erzielten variierende Resultate bei ihren Untersuchungen über den Einfluss des Pervitins auf die Atmung bei der Ratte und beim Meerschweinchen. Insbesondere bei der Ratte wirkte das Pervitin in erster Linie anregend auf die Atmung, aber beim Meerschweinchen war der Einfluss meistens lähmend.

Beim Menschen wirkt das Pervitin atmungsanregend, aber andererseits ist auch beobachtet worden, dass es oft zu einer periodischen Atmung vom Typus Cheyne-Stokes führt (6). Bei durch Morphin und Schlafmittel erzeugten Vergiftungen wirkt das Pervitin, wie wahrgenommen worden ist, antagonistisch (19, 22).

Die Wirkung des Pervitins auf die durch Morphin herabgesetzte Atmung des Kaninchens haben wir in 11 Versuchen untersucht. Aus den Versuchen ergab sich deutlich, dass die einzelnen Tiere individuell sehr unterschiedlich auf das Pervitin reagierten. In einigen Fällen wurde die Atmung unter dem Einfluss des Pervitins schlechter.

Als den Kaninchen das Pervitin in Dosen von 1 mg/kg verabreicht wurde, wurde das Atemvolumen anfangs kleiner, stieg dann aber wieder auf das Niveau, auf welches es durch das Morphin herabgesetzt worden war.

In fünf Versuchen gaben wir den Tieren 2 mg/kg Pervitin intravenös. Zweimal stieg die Atmung auf das normale Niveau, zweimal stellte sich nur schwache Atmungszunahme ein, und einmal wurde die Atmung anfangs schlechter, um später wieder etwas besser zu werden, ohne jedoch das normale Niveau zu erreichen.

Bei drei Versuchen wurde den Kaninchen 3 mg/kg Pervitin verabreicht. Hierbei wurde eine starke, atmungserregende, periodische Wirkung festgestellt. Das Atmungsvolumen stieg anfangs auf das normale Ausmass, sank dann wieder auf das durch das

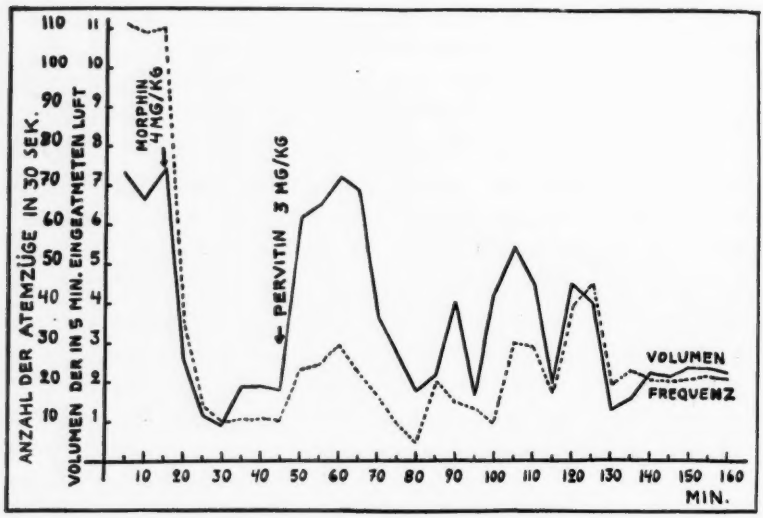

Abb. 6. — Die Wirkung des Pervitins auf die durch Morphin herabgesetzte Atmung des Kaninchens. Gewicht des Tieres 2.45 kg. Morphinhydrochlorid 4 mg/kg intravenös und danach Pervitin 3 mg/kg intravenös.

Morphin herabgesetzte Niveau und stieg wiederum an (Abb. 6). Die Atmungsfrequenz variierte stark, indem sie 3—30 in einer halben Minute betrug. Eigentliche Krämpfe kamen nicht vor, aber es zeigte sich eine gewisse motorische Unruhe. Zwei Kaninchen erhielten 6 und 8 mg/kg Pervitin. In beiden Versuchen nahm die Atmung zu, aber gleichzeitig traten starkes Zittern und klonische Krämpfe ein.

Die Wirkung des Pervitins war bei unseren Versuchen ungleichmässig und hielt gewöhnlich 30—90 Minuten an. In manchen Versuchen hatte die Atmung den Typus Cheyne-Stokes, obwohl dies in den Abbildungen nicht deutlich zu sehen ist (z.B. Abb. 6), da die Fünfminutenserien die Periodizität ausgleichen.

ZUSAMMENFASSUNG

Die Untersuchung hatte den Zweck, die Wirkungen von verschiedenen atemerregenden Stoffen auf die durch Morphin (4 mg/kg intravenös) herabgesetzte Atmung des Kaninchens miteinander zu vergleichen.

Von den untersuchten Stoffen war die das Atmungsvolumen vermehrende Wirkung beim Pikrotoxin und Lobelin am stärksten sowie beim Strychnin am schwächsten. Mit Koffein und Pervitin wurde desgleichen ziemlich starke Erhöhung des Atmungsvolumens erzielt, obwohl der Effekt des Pervitins schwankend war, und bei manchen Versuchen Atmung vom Typus Cheyne-Stokes auftrat. Am längsten hielt die Wirkung beim Pikrotoxin und Pervitin an, nämlich jeweils 30—90 Minuten. Die Wirkung des Strychnins und Koffeins dauerte ca. 20—60 Minuten, und am kürzesten war die Wirkung des Lobelins, welche nur 5—10 Minuten lang anhielt.

Unseren Versuchen gemäss müssen zur Erzielung einer effektiven atemstimulierenden Wirkung alle die von uns untersuchten Atmungsstimulansen in Dosen angewandt werden, die krampfverursachenden Dosen nahekommen oder sonst toxisch sind.

LITERATURVERZEICHNIS

1. Biberfeld: Arch. ges. Physiol. 1904:*103:*266.
2. Blume, W. und Zöllner, R.: Arch. exper. Path. u. Pharmakol. 1943:*202:*21.
3. Bock, J.: Heffters Handb. exper. Pharmak. 1920:II:1.
4. Eckstein, A., Rominger, E., und Wieland, H.: Ztschr. Kinderh. 1921:*28:*218.
5. von Euler, U. S., Liljestrand, G., und Zotterman, Y.: Skandinav. Arch. f. Physiol. 1940:*83:*132.
6. Gross, Fr., und Matthes, K.: Arch. exper. Path. u. Pharmakol.: 1947:*204:*57.
7. Hauschild, F.: Arch. exper. Path. u. Pharmakol. 1939:*191:*465.
8. Hess, J.: Arch. exper. Path. u. Pharmakol. 1941:*197:*204.
9. Heymans, C., Bouckaert, J. J., und Dautrebande, L.: Arch. internat. pharmacodyn. 1931:*40:*54.
10. Higgens, H. L., und Means, J. H.: J. Pharmacol. & Exper. Therap. 1915:*7:*1.
11. Järvinen, P. A., und Vartiainen, A.: Ann. Med. exper. et biol. Fenniae 1949:*27:*78.
12. Koppanyi, T., Linegar, C. R., und Dille, J. M.: J. Pharmacol. & Exper. Therap. 1936:*58:*199.
13. Kossa: Ung. Arch. Med. 1892:*1:*24. zit. nach Koppanyi, Linegar und Dille 1936.
14. Lindhard, J.: J. Physiol. 1911:*42:*337.
15. Liljestrand, G., und Nylin, G.: Acta physiol. scandinav. 1940—41: *1:*328.

16. MALONEY, A. H., FITCH, R. H., und TATUM, A. L.: J. Pharmacol. & Exper. Therap. 1931:*41:*465.
17. LE MESSURIER, D. H.: J. Pharmacol. & Exper. Therap. 1936:*57:*458.
18. NORRIS, V. H., und WEISS, S.: J. Pharmacol. & Exper. Therap. 1927: *31:*43.
19. PÜLLEN, C.: München. med. Wchnschr. 1939:1003.
20. SCHOEN, R., und DERRA, E.: Arch. exper. Path. u. Pharmakol. 1928: *133:*257.
21. TRAVELL, J., und GOLD, H.: J. Pharmacol. & Exper. Therap. 1935: *53:*169.
22. ZUR VERTH, C.: Deutsche med. Wchnschr. 1951:*76:*806. und Slg. Vergift.fälle, Arch. Toxikol. 1952:*14:*229.
23. WIELAND, H.: Ber. d. dtsch. chem. Ges. 1921:*54:*1784.
24. WIELAND, H., und MAYER, R.: Arch. exper. Path. u. Pharmakol. 1922:*92:*195.
25. WHITEHEAD, R. W., und ELLIOTT, D. C.: J. Pharmacol. & Exper. Therap. 1927:*31:*145.